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Edited by

J. Walter Wilson

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Experimental Hepatoma Conference [1,2]

It is the stated function of the Study Section of the United States Public Health Service to promote, develop, and correlate research. This obviously involves more than the appraisal of applications for grants. In discharging its other responsibilities the Morphology and Genetics Study Section has undertaken to hold conferences on subjects that are of wide general interest and that are being studied from a number of points of view so that correlation of effort might be stimulated.

In the field of cancer research the skin has played a considerable role because of its accessibility and the mouse mammary gland because of the complexity of its causation. In our study of grant applications we have been impressed by the number of laboratories devoting all or a substantial part of their efforts to the study of the experimental hepatoma—particularly in rodents. There are several obvious reasons for this. The liver cell is unquestionably the best known somatic cell. To the cytologist and to the enzymologist it has almost come to be the typical cell. The structure and function of the liver are known from many points of view and are amenable to experimentation. Its response, for example, to dietary changes, endocrine changes and to hepatotoxic agents has been extensively investigated. Its regeneration capacity is almost unique as an experimental tool. Tumors can be produced by chemical carcinogens, and by certain dietary regimens, or they may occur spontaneously in certain strains. They also may be transplanted with all the possibilities of studying tumor-host relationship under controlled conditions. This has all led to a great variety of experimental studies which are advancing to a point when a synthesis is possible and desirable. It is not the intention of this Conference to accomplish this synthesis, but it is our hope that it will lay the groundwork on which such a synthesis may eventually be achieved.

J. Walter Wilson

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[1] Received for publication December 14, 1954.

[2] Requests for reprints of the complete Conference should be addressed to Dr. J. Walter Wilson, Brown University, Providence, R. I., and for individual papers to the senior author. This Supplement is also on sale at the Government Printing Office and may be purchased by writing to the Superintendent of Documents, Washington 25, D. C.

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Dr. J. N. P. Davies, Makerere College, Uganda

The Origin of Some Inbred Mice. II. Old Techniques and New [1,2]

LEONELL C. STRONG, *Roswell Park Memorial Institute, Biological Station, Springville, N. Y.*

The origin of many of the inbred mice used in the scientific laboratories of the world was originally a personal endeavor for the purpose of investigating the biological or genetic nature of cancer. It soon became apparent that other investigators appreciated the value of using such mice. This interest was gradually spread to institutions and, more recently, to efforts of maintaining these animals for future science.

Many papers have been published on the origin of certain inbred strains as well as to point out and emphasize the value of using these animals, not only in cancer research but also in other aspects of scientific research. It has been maintained that too many of these strains are being continued—leading to much confusion and to improper observations when not fully understood or appreciated.

There are several purposes in writing the present paper. It is the intention, first, to discuss briefly the principles involved in the origin of some of the original inbred strains of mice. Secondly, it is the intention to discuss more at length some of the new techniques which have been employed recently in the development of new strains of inbred mice. Finally, fundamental principles involved in the origin and use of inbred strains and the principles involved in the continuation of such material so as to benefit quantitative research in the future will be discussed. Their value to cancer research specifically and to general biology and other sciences in general will be reemphasized. There is no attempt here to catalogue the inbred strains now being continued in the various laboratories. This is adequately being taken care of by the Roscoe B. Jackson Memorial Laboratory, and *The Mouse News Letter*, collated by Dr. T. C. Carter, and published in London. Nor is the origin of other strains developed by many investigators discussed or even enumerated. We are concerned primarily with principles.

Old Techniques

When the present author was a graduate student at Columbia University, the fundamental principles involved in the introduction of quantitative research to biology was a critical problem. The introduction of these methods to biology largely stemmed from the science of genetics under the stimulating influence of T. H. Morgan. At that time, the author attempted the application of quantitative methods to cancer research. In looking over the available material, it soon became apparent that there were no inbred strains of animals in existence. The nearest approach to this situation was the dilute-brown strain which had been developed by C. C. Little. During the summer of 1919 a severe epidemic of mouse paratyphoid completely wiped out this pedi-

[1] Presented at the Conference on Experimental Hepatomas, Harriman, N. Y., October 11, 1954.

[2] This investigation has been made possible, in part, by a grant from the Committee on Growth of the National Research Council acting for the American Cancer Society.

greed stock, housed at the Carnegie Institution of Washington, Cold Spring Harbor, New York. During the end of the summer a trio of dilute browns was received at Cold Spring Harbor from Professor Tyzzer of Harvard University, who had kept the colony of these mice for several years. These mice were, as far as the author knows, quite unpedigreed and they were probably in the neighborhood of, at least, a year of age. Dr. Little turned these mice over to me and I was able to continue them by pedigreed inbreeding for several years at St. Stephen's College, Annandale-on-Hudson, New York (now Bard College). After several years, a culture of these mice was returned to Dr. Little and from this source the well known dilute-brown mice have descended.

Another partially inbred colony of mice at the Carnegie Institution of Washington, Cold Spring Harbor, New York, was the so-called Bagg albino which was introduced into the above laboratory by Dr. Halsey J. Bagg following the epidemic of mouse paratyphoid. At that time the Bagg albinos were not being continued by pedigreed brother × sister matings, but by pen matings. It was Dr. Bagg's opinion, expressed to the author in 1919, that pen inbreeding merely meant the nonintroduction of foreign mice. That this system is inadequate to produce inbred mice was shortly evidenced by crosses of the Bagg albino with the dilute-brown mice, mentioned previously. This outcross produced mice in the F_1 of black agouti, brown agouti, black nonagouti and brown nonagouti. About this time some other albino mice of unknown origin were available, and these were outcrossed to the Bagg albino in order to produce sufficient numbers of mice for immediate use.

The original outcross between the dilute-brown and this mixed albino strain (which later by pedigreed brother × sister matings became established as the Strong A strain) served as material for the work on the genetic analysis of susceptibility of the transplanted tumor (*1*). When this study of the transplantation of tumors was completed, the various surviving mice were separated into sublines according to their color combinations. They have been continued as independent sublines up to the present time. These are the well known C3H, CBA, C,[3] CHI, and the less known but interesting $C_{12}I$. Thus only the genetic tools of hybridization and selection were used in the development of many of the inbred mice now in the laboratories of the world. Various degrees of susceptibility to spontaneous tumors (especially the adenocarcinoma of the mammary gland) were developed in these various sublines by selection toward early and late appearance of tumors. The CBA were originally produced by selection toward longevity. In addition to these inbred strains which have had a common origin and still possess many biological and genetic characteristics in common, several other strains of independent origin were established between the years of 1920 to 1927, and have been continued to the present. These are the F, I, JK, L, and N.

The origins of the above mice were discussed in a paper published in *Cancer Research* in 1942 (*2*). Their use in biological research is ever-increasing, first, in cancer research where quantitative biological research on mammals was early appreciated, and second, in practically all fields of general biological interest.

These mice, as well as many others developed by many investigators throughout the world, are now being used in such numbers that it would be quite impossible to list them in a paper of this length. They have proved their value continually. Reproducible results are constantly being obtained. Such diverse observations as sensitivity to estrogenic hormones, nutritional requirements, hemoglobin levels, sensitivity to salicylaldehyde, etc., have been investigated, and what is important is that the observations made can be verified by any capable investigator who desires to repeat the experiment. Many investigators are content to determine strain differences. Many of these differences could probably be put upon a genetic basis if the investigator would continue his observations beyond the F_1 of a cross between mice which differ in any of the above or any other biological characteristic. The determination of strain differences does not solve a problem; it merely indicates that a further analysis is called for.

The use of these strains has made possible observations of a fundamental nature that

[3] Not to be confused with strain BALB/c sometimes referred to as "C."

may not have been made possible otherwise. At least the use of these mice has considerably hastened the observation. One of the most interesting and valuable of these observations in cancer research was the discovery of the milk influence on adenocarcinoma of the mammary gland by J. J. Bittner when he was at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. Another observation in the genetics of cancer susceptibility was the discovery of specific genes involved in the transplantation of neoplastic tissues. This work has been considerably investigated by Little, Strong, Cloudman, and Bittner, and more recently expanded by Gorer, Snell, and other scientists. This work has led to the determination of many so-called histocompatibility genes, and many observations of a fundamental biological nature on their location in the germ-plasm have been very greatly extended, particularly by the monumental work of Snell. This work has been made possible by the availability of many genes involving coat color whose location in the germ-plasm has been determined by linkage studies. The more recent work on adaptations within transplantable tumors by Barrett, Hauschka, Strong, and others stems from the use of inbred mice. This problem, however, is far from being solved and it may very well be that genetic relationships or genetic antagonisms are involved in the so-called "adaptations." Such an analysis is only possible by the use of inbred animals.

This list of contributions could be greatly extended but this need not concern us here.

New Techniques

In addition to the older techniques of hybridization and selection, new techniques for the development of other inbred strains have been introduced. The first of these was the introduction of the carcinogen, methylcholanthrene, into a colony of mice; the second technique was the introduction of the concept that selection based upon parental age may influence characteristics of the offspring. The nature of this influence and the mechanism involved are still quite nebulous. The first of these new techniques for the origin of inbred mice to be discussed will be the injection of methylcholanthrene.

Methylcholanthrene

There are two ways by which the mutagenic property of any agent can be investigated in mammals. These are fundamentally the same but differ in detail. The first consists of the use of highly inbred mice where genetic homozygosity has reached a high level; secondly, the use of mice following an outcross where certain marked genes are in the heterozygous state. Biologically there should be no difference in these two techniques, except that it is easier to detect a recessive mutation if it occurs in the heterozygous individual. Even with pedigreed brother × sister matings from the homozygous state, a recessive mutation could be carried for many generations without manifesting itself. In other forms, Muller maintains that "most mutant genes never become homozygous, since a particular mutant must pass through hundreds of generations before uniting with a similar gene" (*3*).

The present experiment on the injection of methylcholanthrene into mice has been discussed in a series of publications (*4–13*). The experiment consisted primarily of an outcross between two mice of two highly inbred strains, the CBA and the N and, shortly after, a second outcross to a mouse of the JK strain. The ensuing offspring were divided into several sublines, as was done in the old experiment of 1920. These sublines were divided according to the mutant genes that were known to have been present in mice of the ancestral stocks for at least 20 years of inbreeding. One milligram of methylcholanthrene dissolved in 0.1 cc. of sesame oil was injected subcutaneously into the right flank of all mice of the various descents at 60 days of age. This was continued between the F_4 and the F_{40} generations up to the present, or until the time when the particular descent was lost by the acquisition of some biological characteristic incompatible with the continuation of the subline, such as sterility and precocious adiposity. Some of the mutant sublines, however, had to be discontinued on account of inadequate space in the laboratory. It was found after several generations of this continued injection of methylcholanthrene into the parents that many new

color characteristics and other new biological characteristics were being obtained in these mice. After discounting the appearance of many recessive color mutants, which could have been due to delayed segregation of pre-existing genes, it became apparent that many of these new mutants were associated with the injection of methylcholanthrene. On several occasions when the potential parents had been tested against the possibility of their not carrying recessive genes, the recessive mutation was then concluded to be associated with this experimental treatment. This condition of proof that the parent did not possess a marked recessive gene had been fulfilled with a mutation from *P* to *p*, which occurred once in an established subline, and from *S* to *s*, which occurred several times in several independent sublines. Of course, all dominant mutations, such as two dominant spotting ones (of which one was at the *w* locus and the other not-*w*), pintail, and other tail abnormalities (at least seven times), and polydactyly (at least three times) were new and therefore could be considered associated with the introduction of methylcholanthrene. Polydactyly is included with the dominant mutations, but it must be kept in mind that its appearance in the F_1 generation of an outcross to the C57BL strain is only 2.12 percent (observations on about 2,100 F_1's).

In keeping with genetic principles, no recessive mutant is considered to be associated with methylcholanthrene or one of its metabolites unless it did not appear for at least three generations following treatment of the parents. Likewise, it was also the practice to continue a new mutant subline for three generations before attempting to analyze the genetic situation. This second procedure was not necessary, however, since it was soon found that no new mutations were ever detected when the new mutant subline was continued without further injection of methylcholanthrene.

So many mutations had occurred in this one experiment following the injection of methylcholanthrene into both parents for many generations in mice which were all originally genetically related to each other, that it became vital to consider the possibility that the new mutations were due to some peculiar genetic phenomenon such as genic instability. To enumerate all the mutants which have been continued in order to test this possibility of genic instability would again consume too much time. It can be said, however, that in every case of a mutation occurring in the descent of mice exposed to methylcholanthrene a new subline was established immediately and the subline containing the mutant was continued for many generations; the descent, however, was then freed completely from the further injection of methylcholanthrene. In all cases tested, no subline of a new mutation ever gave rise to a subsequent mutation either at the same locus or at any other locus—in spite of the fact that adequate numbers of mice and an adequate number of generations were involved in order to analyze the true genetic situation. As a general conclusion it may be stated that the mutations that were obtained in the generation were associated with the presence of methylcholanthrene or one of its metabolites and were not due to "*genic instability.*"

Only two examples will be mentioned here. In the subline where *P* had mutated to *p*, the mice were continued for twenty generations by mating the mouse of the formula *pp* to a mouse of the constitution *Pp*. This combination continued to produce a 1:1 ratio at the *p* locus and no other mutations were obtained (*14*). In this publication, 641 mice were reported, of which 323 were dark-eye and 318 pink-eye. Expected 1:1 ratio is 320.5 dark-eye : 320.5 pink-eye. Subsequently in this descent 24,541 mice were produced. In this experiment, since the mutation rate in other sublines treated with methylcholanthrene had been shown to be 1 to 750 mice it must be concluded that the original mutation from *P* to *p* was unique and, as far as could be detected, was not associated with any genic instability. The segregation between *P* and *p* was normal following the appearance of *p*, and no other disturbances at the same locus or at other loci could be detected.

In another subline, 3 mutations occurred within two generations treated with methylcholanthrene. These were a mutation from *b* to *B*, *S* to *s*, and *C* to c^{ch}. This multiple mutant subline known as the Blcsp was continued for 15 generations with a population of 18,923 mice without a single new mutation being observed. There

were no reversions nor any genic phenomena that could be interpreted as manifestations of "genic instability." Thus, it must be concluded that 3 independent mutations occurred at 3 independent loci in association with methylcholanthrene or one of its metabolites and these were probably not associated in any way with genic instability. The reasoning involved here is that if genic instability was responsible for the origin of the mutations obtained following the subcutaneous injection of methylcholanthrene, then genic instability should not become "impotent," when the descent had been freed from the further injection of methylcholanthrene.

The mutants obtained in this series following the subcutaneous injection of methylcholanthrene over a number of generations are not unique. They have occurred at old loci which have appeared in the past under spontaneous conditions and, perhaps, at new loci which have not been fully investigated. Many of the biological characteristics which have occurred in these series of mice treated with methylcholanthrene, such as precocious sexual maturity, excessively large first litters, precocious adiposity, gigantism, somatic mosaicism, etc., have not been put on a genetic basis. These have been obtained as well as significant deviations of susceptibility to neoplastic disease. Some of these are genetic, while others may be nongenetic. This aspect of the problem has been discussed elsewhere (*15*). It was pointed out in this publication that instead of waiting 10 or more years to develop relatively pure strain mice by the old techniques of hybridization and selection alone, one can, with the introduction of methylcholanthrene and possibly other agents, obtain variations in degrees of susceptibility to neoplastic disease within a relatively short time. The establishment of an inbred strain of mice possessing an hereditary gastric lesion following its original induction by methylcholanthrene is of prime genetic significance, no matter what the final pathologic opinion of it proves to be (*16–18*).

The above observations indicate that with methylcholanthrene the tempo of biological and genetic change can be considerably increased, above the rate usually associated with spontaneous conditions. In fact, it may very well be that methylcholanthrene or one of its metabolites is the best mutagen for mice that has so far been discovered.

Litter Seriation

A new technique for the development of other inbred mice was recently introduced into the laboratory following the observation that the age of the mother at the time the offspring were born had an influence on susceptibility to chemically induced fibrosarcomas (*19–32*). The observation of a similar nature had also been made in the inheritance of polydactyly in mice. Some older observations in the literature, such as polydactyly in guinea pigs by Sewell Wright had not led to the introduction of this technique for the origin of new strains of experimental animals.

In contrast to the two techniques for the origin of inbred mice, as outlined above, where hybridization was first resorted to and then the establishment of sublines by selection, with and without the introduction of methylcholanthrene, the new technique starts with the use of mice of already established inbred origin. It must be clearly kept in mind, however, that the degree of homozygosity that any mice possess is only a relative value. The theoretical limit in which 100 percent of all genes are homozygous is never reached. It is also clear that spontaneous mutations do occur and these would naturally increase heterozygosity. In scientific laboratories where relatively few numbers are and can be supported, this factor of mutation is not too serious. In large colonies, particularly used for commercial purposes, since spontaneous mutation rates are as they are, elimination of all collateral or side lines, is a very serious problem. The estimation of mutation rates in mice differs considerably in different laboratories. In my own colony of approximately 450,000 over a period of 35 years, a spontaneous mutation rate of visible mutants has been approximately 1:26,000 mice. The spontaneous mutation rate of so-called invisible mutations in mice such as lethals, semilethals, etc., is quite an unknown factor. Some attempt to analyze these invisible mutations has been made by Snell, Russell, and other investigators. H. J. Muller, in a recent article in *Modern Medicine* (*3*), points out from evidence obtained from

radiation that mice are even more sensitive to radiation (6 or 7 times) than *Drosophila* and other forms of life.

The biological effects that can be measured by litter seriation or parental age are of small magnitude. Some evidence, although not exhaustive, due primarily to the newness of the observation, indicates that these fluctuations are cumulative over a number of generations. Thus divergence in biological characteristics, such as susceptibility to chemically induced fibrosarcomas and polydactyly in mice, can be obtained by beginning with a relatively homozygous constitution and basing selection only upon the age of the mother at the time the offspring were born. In both series (chemically induced tumors and polydactyly) selection is being continued in the parents without recourse to methylcholanthrene or any other experimental procedure.

The inheritance of multiple factors cannot be excluded entirely in the present selected sublines, but it is emphasized that the offspring are mated together for the production of the next generation before the complete potential of the parents can be determined. Thus, a selection based upon either the progeny test or the percentage of incidence of polydactyly in the offspring, which should vary with the accumulation or suppression of multiple genes, is carefully avoided.

Another point of biological or genetic interest should also be kept clearly in mind. It is still not clear whether this divergence in a biological characteristic based upon selection of parental age is permanent to the strain or only of a temporary nature. In Cladocera, Lansing maintains that the effect of maternal age on longevity is both cumulative and reversible. It may be that divergence is merely of a temporary nature in order to prevent a species from drifting into chaos by the accumulation of an aging factor from the parents. Compensatory biological changes are possible. It is, therefore, premature to discuss the fundamental nature of this divergence brought about by selection based upon litter seriation or parental age and the mechanism involved in its origin. For the time being, it is the intention merely to point out a new tool of selection based upon the concept of parental age, which is producing new strains of mice which differ significantly in measurable characteristics from mice of other inbred strains.

General Discussion

The Roscoe B. Jackson Memorial Laboratory has done a herculean task in maintaining and making available to other investigators, mice of the various inbred strains developed not only by themselves but also by many other geneticists throughout the world. It is not generally recognized how much time and effort is required to produce mice of uniform genetic background. It is indeed fallacious to assume, as many do, that brother × sister matings will continue the uniformly isogenic state, particularly when mice raised for commercial purposes are produced by the thousands. It becomes absolutely necessary to restrict descent by discarding all mice with collateral lines even though they be descended from the same original stock. This is particularly difficult to do in view of the fact that, commercially, it becomes sound to raise as many mice as possible under the conditions in which they are being produced. On a general average, mice of the pedigreed strains do not breed so well as mice of hybrid sources and they are subject to more of the common laboratory diseases.

Our Federal Government, through the interest of the National Institutes of Health, has also initiated a program for the continuation of some of these pure strain mice.

What is needed now is the establishment of several other similar laboratories throughout the United States so that the availability of the mice is assured to investigators without having to resort to long express travel of the animals, which is not conducive to the best interests of the animal. As a general rule it is perhaps best to experiment upon a small rodent, such as a mouse, in a laboratory in which the animal has been born. This is particularly true in such fields as cancer research where susceptibility to the various kinds of neoplastic diseases is very delicately determined and can be altered significantly even by transferring an animal from one laboratory to another. This experience has occurred several times, particularly in mice of my

own colony which were shipped from Boston, Massachusetts, to Ann Arbor, Michigan, and in the colony of Marsh which was sent from Springville, New York, to London, England, several years ago. In each case, it required several months for a given level of susceptibility for adenocarcinoma of the mammary gland to re-establish itself after the transfer.

The commercialization of pure-strain mice is another problem of widespread interest and importance. Some of the antiquated concepts for the raising of experimental mice are gradually being replaced by more modern ones. Some dealers, however, admit that their colonies are being continued by pen matings, which merely means the nonintroduction of foreign mice, and it has been proved that such a system will not continue pure-strain mice, particularly when they are raised by the thousands. Even brother × sister matings will not continue purity unless drastic elimination of all sublines is periodically resorted to, and this the commercial breeder is loath to do particularly when he must produce large numbers in order to keep in business. However, much improvement is being accomplished by some of the commercial raisers who appreciate modern genetics and are trying their best to produce experimental mice in adequate numbers to satisfy customers. It costs more to produce pure-strain animals, and this item must be appreciated and paid for by the investigator.

One of the most serious drawbacks to the use of pure-strain mice is the fact that some of the original inbred strains have now been broken up into an indefinite number of sublines. These various sublines are of value to a geneticist, who is interested in the analysis of the factors involved in the origin of these variations, but are quite confusing to a chemist or even an endocrinologist. Perhaps it would be well either to restrict considerably the number of sublines which have been developed from one original source or to give the various sublines different symbols. For example, when the present author arrived in Springville the well-known DBA stock was represented in 176 sublines all of which had been kept distinct for several years. These mice differed considerably in many biological characteristics such as the occurrence of spontaneous adenocarcinoma of the mammary gland and their ability to grow transplanted tumors that had arisen in strain DBA. One of the first duties and pleasures of the new director of the biological station was the elimination of these impossible collateral sublines.

It has been maintained also that there are already too many inbred strains of experimental animals in existence. Why bother therefore in the development of new? This reasoning is fallacious. In the first place the introduction of new techniques for the development of new strains of inbred mice as outlined in the present paper is introducing new biological concepts of a fundamental nature, and, as such, justify themselves. In the second place, the types of tumors obtained and the many biological characteristics possible which can be stabilized by the present methods of genetics are still small. All too few neoplastic diseases occur under controlled genetic conditions. The possibility of obtaining other characteristics and other tumors under controlled conditions appears to be unlimited. Instead of having too many inbred strains, we still do not have enough. The facilities to carry on this wealth of biological material are still inadequate. The inbred strains should not be exposed to the vagaries of commerce but protected by a better or more substantial system.

One concept that arises from the task of determining genetic or biological relationships between mice of different inbred strains should be of interest to the present group. It is now well known that mice both of the C3H and CBA strains develop spontaneous hepatomas. The opinion has been expressed for some time that hepatomas were arising in two separate strains of mice. When one analyzes the origin of these two strains, it is apparent that they had the same origin and must retain many genetic and biological characteristics in common. Thus the spontaneous hepatomas that are arising in mice of these two strains may be due to certain genetic or biological identities. Again many of the physiologic responses of mice to a variety of experimental procedures such as the introduction of estrogenic hormones are similar. Many of these similarities are due not to experimental variability but to genetic or

biological relationships. It is not always clear whether one is dealing with an agent that transcends all genetic determination or merely to the identification of transmission of genetic determiners in common. Some of these genetic relationships between mice of different stocks are not always clear. Representatives of the various inbred strains have been given to investigators throughout the world. Many of these investigators retain the symbol of the original inbred strain and clearly state in their publications where the mice were obtained. A certain few have actually changed symbols unethically and are always vague in crediting the true origin of the mice.

One of the greatest defects in the use of pure-strain mice is the fact that after a difference between mice of two strains has been determined, the experiment is terminated there or even in the F_1 generation as stated previously. On the surface it looks as though the experimental procedure has actually brought about the biological effect, whereas if the experiment is continued into the significant generations of the F_2 and the backcross to the recessive condition it may become evident that what the experimental procedure has brought about is merely a measure of genetic determination.

The use of inbred experimental animals has more than justified the efforts involved in their origin. Many observations are now being made by their use. Their value continues to increase and their use is becoming almost universal.

Conclusions

Three methods have been employed in the development of mice of pure-strain descent. These are as follows:

A. By hybridization and selection. Selection has been based upon (i) the appearance of spontaneous tumors such as adenocarcinoma of the mammary, (ii) longevity, etc.

B. Hybridization and selection followed for many generations by the injection of methylcholanthrene into breeding mice.

C. Selection by starting with a relatively inbred or homozygous state and basing selection upon litter seriation or the age of parents at the time the offspring were born.

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Dr. J. Walter Wilson, Biology Department, Brown University, Providence, R. I.: At the Zoologists' Meetings in Boston, Dr. Elizabeth Russell reported a study being conducted at The Jackson Memorial Laboratory on the occurrence of tumors in various strains of mice at the end of their normal life-span. At my request she submitted the following data and comments concerning the incidence of hepatomas:

Autopsy data on 1,278 breeding females and 791 males from 10 major strains, all born in the Inbred Nucleus between January 1, 1948 and January 1, 1951

Two strains had no hepatomas:
AKR: 0/65
BALB/c: 0/261.
Four strains had very rare hepatomas:
A/Jax: 1/65
A/HeJax: 2/257
DBA/1: 1/249
DBA/2: 1/253.
A slightly higher incidence characterizes the three C57 lines:
C57BL/6: 18/444
C57BR/cd: 8/127
C57L/He: 8/88.

In the C3H strain, hepatomas are extremely rare in females (1/223) and frequent in males (35/138, or 26%), increasing in prevalence with advancing age (40% of males over 400 days).

For an age analysis, only the C3H and C57BL/6 have been sufficiently studied. The average age of animals with hepatoma is less for the C3H strain than for the C57BL/6:

Average life-span of C3H males=424 days.

Age at death of 36 C3H males with hepatoma=200 to 800 days; 31 of them were over 400 days.

The single C3H female with hepatoma died between 250 to 300 days, and had a mammary cancer as well as the hepatoma (average life-span of C3H breeding females=314 days).

Thus it is possible for young C3H females to have hepatoma, but most of them die before the age at which hepatomas become frequent in C3H males. In addition to this, hepatomas definitely can appear earlier in C3H than in other strains.

The C57BL/6 is a very long-lived strain, mean life-span of females, 563 days, mean life-span of males, 529 days. However, even within this long life-span, hepatomas are late in appearing.

In C57BL/6 females, animals with hepatomas were over 500 days of age, although 80 younger females were studied in detail.

In C57BL/6 males, all animals with hepatomas were over 600 days of age, although 83 younger males were studied in detail.

Histopathology of Carcinogenesis and Tumors of the Liver in Rats[1]

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Ever since 1932 when Yoshida (*1*) first reported the induction of tumors of the liver in rats with *o*-aminoazotoluene, experimental tumors of the liver have been one of the chief objects of study in cancer research. The composition and purposes of this Conference exemplify the scope and depth of such studies. I plan to describe some of the anatomical and biological characteristics of these tumors from their inception to their full development. Others on the program will discuss the chemical, dietary, hormonal and other factors important in the pathogenesis of these tumors.

In the field of morphology the tissue changes during the induction of liver tumors in rats with butter yellow were well described in the late '30s and early '40s by several investigators (*2–8*). Subsequently, various derivatives of butter yellow were used (*2, 4, 9, 10*). Also as early as 1944 Bielschowsky (*11*) obtained tumors of the liver in rats using 2-acetylaminofluorene.

Much of the material which I will describe and illustrate is taken from studies on rats carried out in collaboration with Dr. Harold Morris or with Dr. Mulay, both at the National Cancer Institute. I will touch only very briefly on material in mice and men, and not at all on the tumors in dogs and other animals.

In studies with Dr. Morris, rats were fed either acetyl- or diacetyl-aminofluorene in diets low in choline, protein and fat, and high in carbohydrate (*12*). In those with Dr. Mulay rats were fed either butter yellow or a butter-yellow derivative, dimethyl-aminobenzene-1-azo-1-naphthalene (DAN) and a similar diet (*13*). In all these animals the lesions which developed in the liver can generally be divided into two categories, those changes involving principally the biliary ducts and those involving mainly the hepatic parenchymal cells and lobular structure. Actually, in almost all instances, changes in the biliary tree and hepatic parenchyma developed concurrently and this separation is solely for the purpose of description and in no way is meant to detract from the importance of their interrelationships. I will not attempt to date these lesions specifically because the rate of their development varies so much. Demonstrable tumors can be obtained as soon as 6 to 8 weeks, or they may take well over a year, depending on the carcinogen, the dose, the diet, and other factors.

Histogenesis of Cholangiomas and Cholangiocarcinomas

The earliest changes in the biliary tree consisted of proliferation of small bile ducts as thin strands of spindle-shaped cells in a double row with elongated oval nuclei arranged in tandem extending from the portal areas into the adjacent parenchyma (fig. 1). Generally the lumen was either collapsed or as yet unformed. This lesion, as it became more advanced, was accompanied by slight portal fibrosis and there was

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 11, 1954.

accentuation and, at times, also some distortion of the lobular architecture. Macrophages containing brown ceroid pigment were seen in the portal areas.

A number of studies have been made of this lipofuscin pigment called ceroid (*7, 14–16*). It is acid-fast, periodic acid-Schiff positive and strongly fluorescent in ultraviolet light. The present concept is that it represents unsaturated lipid which undergoes auto-oxidation and polymerization to a yellow amorphous substance changing to brown. It is insoluble in fat solvents and therefore stainable with fat stains in paraffin sections. Its formation can be prevented transiently by adequate administration of the antioxidant, vitamin E (*17*).

Thus far, all the changes I have described are those of dietary cirrhosis which can be observed in animals on choline-deficient diets alone (*14*). White and Edwards (*18*) noted that with the addition of carcinogen to the diet some of the bile ducts became distended and cystic. In the material which I have studied these cystic ducts become distended with clear yellow or yellow-green fluid and are evident grossly as multiple small, multiloculated, clear cysts. Microscopically, in the early stages of their development one can clearly recognize the bile-duct nature of their lining epithelium but as they enlarge and become more distended the lining epithelium flattened and simulates endothelium such as one might find in a lymphangioma. In some instances, it is apparent by studying changes present in the same liver in somewhat different stages of development that the cystic spaces arise from obstructed smaller bile ducts which have grown into the hepatic parenchyma; in other instances larger bile ducts with columnar lining epithelium are involved. There are a few loculated collections of cysts that seem to involve both sizes of ducts (fig. 2). In the case of the smaller ducts and when the cysts are well formed, groups of hepatic parenchymal cells are often trapped in the stroma between the ducts.

These cystic lesions which develop in rats fed carcinogen are fairly numerous and almost invariably benign. They accompany almost all the cases with cirrhosis, yet tumors of bile ducts, such as cholangiocarcinoma, comprise only a small percentage of hepatic neoplasms. Furthermore, if these cystic lesions constituted forerunners of carcinoma, the early stages of malignant transformation would certainly be evident from time to time. Thus it seems evident that transition of these lesions to cholangiocarcinoma, if it ever occurs, must be quite rare. I have seen only one instance suggesting such a transition, out of at least several hundred animals with multiple cystic lesions. It was in the liver of a rat fed acetylaminofluorene. At the margin of one lobe of the liver there was a large loculated cystic mass and at its center, on cut section, there was a more solid growth of cholangiocarcinoma with taller cells and with atypical cytologic characteristics and histologic pattern (figs. 3 and 4).

In a few instances, areas of more solid, noncystic, glandular proliferation occur and may represent benign neoplasms, and such lesions may justify the terms bile-duct adenoma or cholangioma (fig. 5).

Finally, there is a curious lesion of bile ducts described by Opie (*8*) as cholangiofibrosis (fig. 6). This lesion consists of a group of proliferative bile ducts in the portal areas which contain mucus and often a few polymorphonuclear leukocytes. The ducts in these areas are surrounded by a rather dense fibrous stroma.

The relationship of this lesion to carcinoma was first pointed out by Opie, who traced histologic transitions from such areas of cholangiofibrosis into carcinoma. Although so much of the interpretation of transitions in tissue sections is subjective, I too feel that I have observed similar changes. This interpretation was strengthened for me in that the cystic lesions described above are free of mucus in sections stained with the periodic acid-Schiff technique but the mucus, always evident in areas of cholangiofibrosis, is usually readily demonstrable in the cholangiocarcinoma (*19*) (fig. 7). Also, there are areas of mucus, stained deep red in heavy fibrous stroma, visible in some of the tumors even after tumor growth and necrosis had otherwise made them difficult to recognize by ordinary techniques. This suggests the presence of previous cholangiofibrosis in some of the tumors. This interpretation is somewhat tenuous, however, in that mucus production by tumor acini and/or inclusion of

"innocent" areas of cholangiofibrosis by the tumor provide sources for error in this interpretation.

It should also perhaps be mentioned that it is in these areas of cholangiofibrosis that one finds abundant fibrous stroma which also forms a characteristic, though generally non-neoplastic appearing, integral part of cholangiocarcinomas. By contrast, hepatomas of the liver-cell type are not accompanied by similar stroma. Also, because of the frequent presence of mucus in these tumors, I have found the use of the periodic acid-Schiff technique useful in distinguishing cholangiocarcinoma from hepatoma of the liver-cell type. This is true not only in the rat but also in man (*19*).

The periodic acid-Schiff positive, mucinous material in cholangiocarcinoma should not be confused with other similar reacting material in sections of liver. In liver cells and in hepatoma one can often find multiple, spherical, periodic acid-Schiff positive, hyaline bodies in the cytoplasm which are variable in size, homogeneous, and which stain a light red rather than the dark purplish-red of mucin. The mucin is fibrillar in structure and lies either free in lumens or forms a single large globule occupying most of the cytoplasm of the cell. Neither mucin nor the hyaline bodies are affected by previous diastase digestion as glycogen is. For this reason, as well as the morphologic difference, glycogen in hepatoma cells can also be readily distinguished from mucin. Of course, mucicarmine also stains this epithelial mucin in the cholangiocarcinoma, but it is a less brilliant stain and less widely used.

Curiously, in three cholangiocarcinomas arising in rats fed a butter-yellow derivative, *p*-dimethylaminobenzene-1-azo-1-naphthalene, there were areas of squamous metaplasia similar to those which are sometimes seen in adenoacanthoma of the extrahepatic bile ducts in man. Also, it should be mentioned that cartilage and bone sometimes can be found in the stroma of these tumors.

Histogenesis of Hepatomas (Hepatocellular Carcinomas)

The cholangiocarcinomas, with most carcinogens, form a small but definite minority of the lesions. The majority of the tumors are composed largely of liver-cell type of growth. The earliest changes noted morphologically in the livers of the rats depend on a number of factors, not the least of which is the diet. Opie (*8*) and some of the earlier workers using butter yellow and a variety of diets, chiefly low in protein, low in choline, and high in carbohydrate, sometimes found central fatty change with degeneration and necrosis of the parenchymal cells. In our work (*20*) using diacetylaminofluorine and somewhat different diets, with higher protein and choline supplements at intervals, changes in the hepatic parenchyma were characterized by a minimum of cytologic degeneration and only a few foci of fatty change were evident. In rare instances there were foci of acidophilic necrosis but these were so uncommon as to suggest that they were unrelated to the diet or carcinogen and may have represented coincidental infection. Such areas can at times be found in the livers of untreated rats and mice.

Despite the almost complete lack of evident necrosis there was apparent shrinkage of liver cells and nuclei in the central areas. In the periportal areas there was an increased number of cells which at first tended to be small, closely packed, and which contained increased amounts of basophilic material in the cytoplasm (fig. 8). This basophilic material has been shown by ultraviolet absorption, pyronin-methyl green and ribonuclease digestion to represent ribonucleic acid, probably as ribonucleoprotein (*21–23*), and is characteristic of growing and dividing cells. As these areas developed, the nuclei of the cells became larger, the cytoplasm was more abundant and contained much basophilic material. These changes progressed along with the development of cirrhosis, and foci of hyperplasia were evident in many periportal areas. Mitotic figures were uncommon, but double nucleated cells were frequent in the early stages of hyperplasia. Such areas of hyperplasia continued to grow and formed nodules which compressed and distorted the adjacent hepatic parenchyma (fig. 9) and mitotic figures became more frequent. At this stage, distinct nodules were

evident grossly in the liver and generally they were reddish-tan and resembled the surrounding liver tissue in color and consistency.

Somewhat later, nodules were more often found which grossly appeared paler tan, gray, or white. On section of these later, more developed nodules, there was loss of any semblance of cordlike structure, and in the central areas there appeared to be loss of stroma and cohesiveness. Often there were foci of degeneration and necrosis of cells centrally in the nodules, presumably as the result of ischemia and inanition. The cells in these nodules showed increasing atypicality with variation in size, shape, and staining. Nucleoli were large and sometimes multiple. Mitotic figures were often frequent and at times distinctly abnormal. In my opinion, these nodules constitute the earliest definite neoplasms that we can distinguish morphologically (fig. 10).

Still later when large hepatomas of the parenchymal cells developed, occupying most of a lobe or more of the liver, one could see essentially the same cellular and morphologic pattern but just more of it. Many people have transplanted the tumors at this stage and verified their biological neoplastic nature. It has been tumors such as these that have generally been used for many of the studies which we will hear about from the chemists, cytologists, and others at these meetings.

Some of the better differentiated tumors have a trabecular pattern with double cords of cells separated by prominent vascular spaces, forming the so-called "trabecular hepatoma" (fig. 11). Other tumors have a sort of glandular pattern, "adenohepatoma," with pseudoacinar structures consisting of rings of cells resembling hepatic parenchymal cells with large round or oval vesicular nuclei, prominent nucleoli and marginal chromatin lining the nuclear membrane (fig. 12). These nuclei are generally located in the center of the cells without any polarization and the cytoplasm is abundant, eosinophilic, and often protrudes into the central lumen, forming a scalloped pattern lining the pseudoacinus, in contrast to the typically smooth lining of the acini and ducts of cholangiocarcinoma. Some hepatomas of liver-cell type have more basophilic cytoplasm which is occasionally vacuolated and contains fat. In others, the tumor grows as solid sheets of cells. Finally, in any hepatoma all, or any combination of these patterns may be found in different areas of the tumor (fig. 13).

In hepatomas of human origin there is one feature which, except for Kinosita (*2*), has never been reported in those of the rat; that is the secretion of bile into canaliculi between adjacent liver cells. This has frequently been found in the metastases as well as in the primary hepatoma in man. I have also seen it in a metastasis in a supraclavicular lymph node from a teratoma of the testis in which the metastatic lesion contained well-differentiated liver cords (fig. 14).

Problems of the Chemical and Histochemical Analysis

As might be anticipated, the variability in the cell pattern of liver tumors is matched by the variability in histochemical and chemical content of the tumors. Most reports show wide variations in the chemical results from tumor to tumor (*24*, *25*) and the histochemical pattern of such things as glycogen, fat, acid phosphatase, and alkaline phosphatase shows similar variations, even from area to area in the same tumor. In chemical determinations acid phosphatase varies from low to normal values. Generally speaking, in our experience (*25*) alkaline phosphatase is high in regenerating and hyperplastic areas and is always increased in tumors of the liver. However, in studies of sections, the Gomori technique reveals that alkaline phosphatase is located largely in the stroma around bile ducts, in endothelial and Kupffer cells, in necrotic areas, and in liver cells about the bile canaliculi; but frequently the tumor cells are devoid of alkaline phosphatase (fig. 15). As I have already indicated, mucin also varies. Some cholangiocarcinomas contain considerable mucinous material, others very little, and hepatomas, of course, contain none at all.

Then there is the serious problem which faces every chemist who tries to analyze tumors of the liver, both induced and in transplant; that is, the extensive necrosis which may involve all but a shell of viable tumor about the outside. Despite attempts to separate the necrotic and viable tissue, such separations are never complete. For

this reason, Stowell and some of his co-workers at Kansas became interested in the chemical and histochemical changes that take place with time in autolysis, in artificially induced necrosis. They have found that all the enzymes for which they analyzed decreased with time. Generally the oxidative enzymes decreased much more rapidly than the others (*26*). In addition, as one would expect, the nucleic acids and proteins also decreased with time and *in vitro* their breakdown products were found in the trichloroacetic acid-soluble fraction containing nonprotein nitrogen and acid-soluble phosphorus.

Another serious difficulty which confronts anyone attempting chemical analysis of tissues is the heterogeneity of cell constituents. Yokoyama *et al.* (*27*) have shown that in normal mouse liver only 56 percent of the nuclei are from liver parenchymal cells. In regenerating mouse liver, Chalkley counts revealed that anywhere from 10 to 32 percent of the liver was occupied by material other than liver parenchymal cells (*28*). The only similar studies that have been reported on liver tumors were by Stowell (*29*). In butter-yellow-induced tumors of the liver in rats he found as little as 10 percent neoplastic cells and as much as 60 percent connective and vascular tissue, in addition to tissue spaces, intermingled non-neoplastic liver cells and small areas of necrosis.

Cholangiohepatomas

Finally, I must confess that up to this point I have been guilty of over-simplification of the classification of the tumors which one obtains in the livers of rats treated with any of the hepatic carcinogens. Even though I have tried to separate the process of carcinogenesis of liver cells giving rise to hepatomas of liver-cell type from that of bile-duct epithelium giving rise to cholangiocarcinomas, this has been done, for the most part, for the purpose of clarification. Actually, if we examine multiple sections of well-developed tumors of the liver in rats we find, more often than not, that the tumors are composed of both cell types, with large areas of one merging with areas of the other (figs. 16 and 17). Sometimes such cholangiohepatomas are found even in human beings. Of course, the possibility of collision of two different tumors always has to be considered in such a case and, indeed, this possibility cannot be completely discarded. It has been shown that in rats the carcinogen is capable of inducing neoplasia of both cell types. If these are so-called "collision tumors," however, one must assume, I think, that the "collision" took place early in the process of the tumor growth because of the inseparable intermixture. Perhaps three-dimensional reconstructions of serial sections of a number of such tumors might shed some light on this.

My own feeling on this subject is that we are probably dealing with neoplasia of a more primitive cell type, which has the potentiality to develop in either direction (fig. 18). This type of bilateral differentiation in the tumors adds support to the concept of the existence of such cells in normal adult liver unless one wants to invoke the idea of dedifferentiation, which I prefer to avoid.

Similarities of Morphologic Structure of Liver Tumors

The sections I have shown have been from rats fed diets poor in protein and given acetyl- or diacetyl-aminofluorene, butter yellow, or a butter-yellow derivative, *p*-dimethylaminobenzene-1-azo-1-naphthalene. Although I have had no firsthand experience, I gather from the literature that with *o*-aminoazotoluene and other carcinogens the tumors obtained in rats are all similar. With the various carcinogens there are some variations in the percentage of tumors, in the ratio and percent of the cell types involved, and in the speed of induction, but from a morphologic point of view they would appear closely related. Certainly one could not tell from looking at a microscopic section of a given hepatic tumor whether it was spontaneous or induced by any one of a number of carcinogenic compounds. Similarly, one could not tell from the tumor alone what sort of diet was used, what the sex or hormonal status was, or what

strain or even species of animal one was dealing with.[2] This is not unique for tumors of the liver, or for the experimental animal. If we could tell the causative agent by looking at a microscopic section of the tumor, we would probably not be here today.

By contrast, if one examined the liver adjacent to the tumor, one might obtain some clues at least in regard to diet and hormonal status. A low fat, high carbohydrate, low protein diet is prone to produce fatty change in the hepatic parenchymal cells and result in cirrhosis (*14*). In my own experience with the material of Dr. Morris at the National Cancer Institute, we have found in strain A × C rats on a given diet and carcinogen that sex and hormonal status are important; the presence of androgens was conducive to cirrhosis and tumor formation but, in the absence of androgens, cirrhosis and tumors were much less frequent, or absent (*20*). The adrenal cortex and pituitary have also been shown to influence carcinogenesis, but we will leave that for future discussants (*30*, *31*). Also it has been well shown that, in general, high protein diet and, in particular, riboflavine tend to protect against cirrhosis and tumor formation (*32*, *33*). However, this protection, although generally parallel, is not always completely so because some tumors do develop in livers without prior cirrhotic change.

Role of Cirrhosis

What is the role of dietary cirrhosis in the development of tumors of the liver? Certainly there is a high positive correlation between the incidence of the two, both in experimental animals and in man, particularly in cases of hepatoma of the liver-cell type. One might speculate that the "regenerating" areas which form a part of the cirrhotic picture are more susceptible to tumor transformation. I do not know of any experiments on such regenerating areas in cirrhotic liver, but Skoryna and Webster (*34*) did repeated partial hepatectomies on rats given 2-acetylaminofluorene and failed to accelerate or increase the development of tumors. Thus it would seem that true regeneration *per se* cannot be too important.

Opie (*8*) noted a difference in the number and distribution of tumors in the livers of rats with and without cirrhosis. In the cases with cirrhosis, tumors were fewer and were confined to the right upper lappet, presumably where most of the stream of portal venous blood bearing the carcinogen from the small intestines is channeled. In cases of cirrhosis Opie found more tumors and tumors more widely distributed in the liver, presumably because in the abnormal obstruction of the venous flow, the normal channeling of the blood from the intestines was altered, and carcinogen-bearing venous blood circulated through a much wider area in the liver. Thus cirrhosis may cause a much larger part of the liver to be exposed to the carcinogen, and the statistical chances of tumor development therefore improve.

Helen Dyer (*35*) administered butter yellow to rats and reported the slower development of liver tumors in rats on a low choline diet with cirrhosis than in those without cirrhosis on a choline-supplemented diet. At first this seems contradictory but, when we realize that one of the characteristics of cirrhosis is to cause the development of collateral channels of circulation and to cut down the volume of portal venous blood flow through the liver, it becomes apparent that some of the carcinogen that would ordinarily go to the liver is shunted away and therefore, in effect, the dose to the liver is reduced. Admittedly this effect would be submerged if the dose of carcinogen were high enough and it would only be detected by using a marginal carcinogenic dose.

Regardless of how we interpret these findings, cancer still develops in a significant percentage of animals without cirrhosis, and choline, which will protect against cirrhosis, does not prevent carcinogen-induced tumor formation. Therefore, cirrhosis in itself does not play a critical role in the development of tumors of the liver in rats.

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[2] A partial exception is the tumor occurring in the livers of mice, which is almost without exception a hepatoma of the liver-cell type. It is often extremely well differentiated, with normal-appearing cord formations arranged in a radial pattern about a vein. This lobule-like pattern is found not only in the primary tumor, but also in metastases and on transplantation. I know of no other metastasizing tumor of the liver in any other species which is so well differentiated. Of course, at times, undifferentiated areas entirely similar to hepatomas in other species are also seen in this tumor in mice.

It seems that the study of the histogenetic development of tumors of the liver in non-cirrhotic animals would be worth while and less confusing, although the numbers of such tumors would be considerably less.

With respect to changes in the hepatic parenchymal cells leading to the development of hepatomas of the liver-cell type, the succession of findings which I have described corresponds for the most part with the description of Orr (*5*) and with some of those of Opie (*8*). They differ somewhat from the parenchymal changes noted in some of Opie's animals, in which he observed central fatty change preceding the bile-duct proliferation, then edema of what he called septa between the lobules, which I presume was due at least in part to stasis. This was followed by degeneration of cells and fibrosis in the central areas, and regeneration of the peripheral parenchymal cells around the portal zones. Also some fibrous proliferation and new capillary formation were noted in the portal areas. These changes described by Opie correspond to those described by Himsworth (*36*) as diffuse hepatic fibrosis, which characteristically follows fatty change, progressing to cirrhosis due to deficiency of choline and/or riboflavine deficiency. Therefore, I would propose that these anatomical differences for the most part represent the effects of differences in diet. The fact that both observers obtained similar tumors in their animals indicates that such variations do not represent critical differences in the carcinogenic process, but merely serve to complicate the interpretation of anatomical changes occurring in the liver.

Correlation of Morphology and Biological Behavior

Finally, from the morphologist's point of view, and in order to interpret data gathered from studies of earlier stages of regeneration, hyperplasia, and neoplasia in the liver, we need biological confirmation of the morphologic interpretation of the early lesions. For this reason we have planned, and have under way, an experiment to try to establish the biologic characteristics of these nodules which I have termed early tumors.

One needs to establish at what point these nodular growths become irreversible and lose their dependence on the carcinogen, by withdrawing the carcinogen once such nodules have developed as proved by biopsy. In addition, to carry the study somewhat further, and in an attempt to correlate our morphologic findings with the biologic behavior of the tumors, we plan to transplant some of these nodules and other larger, more well-developed tumors into other sites in the same animal and into other animals of the same and different strains, somewhat in the manner that Greene and Newton (*37*) have done with spontaneous endometrial carcinoma in the rabbit. These studies may also give us information about the state of dependency *versus* autonomy of the neoplastic growths.

Summary

Hepatic tumors can be induced in several species of animals by a number of different carcinogens. The resultant tumors cannot be distinguished from one another on the basis of the agent used, although the incidence and rate of development may vary. The tumors in rats and in man can be classified morphologically into either liver-cell type or bile-duct type or a combined variety. The variability of cell type, functional state, content of extraneous cells, and necrosis all pose difficult unsolved problems for chemists analyzing such tumors. Changes induced in the liver by diet tend to complicate the morphogenetic picture, and although diet and hormonal status influence the development of tumors, they do not appear to be the final determining factors in rats.

In the development of bile-duct carcinomas, cholangiofibrosis seems to be the usual precursor, and periodic acid-Schiff stains help to distinguish these tumors from hepatomas.

Some investigators have been impressed by necrosis and regeneration as preceding the hepatomas. In our studies the relative lack of necrosis indicates that it is not a necessary precursor and the proliferative changes would seem to represent hyperplasia

rather than regeneration. Therefore, I interpret this hyperplasia as a reaction to the carcinogen more than a regenerative reaction to previous necrosis. The transition from hyperplasia to neoplasia is gradual morphologically, and there is need for biologic correlation with the morphologic interpretation.

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PLATE 98

FIGURE 1.—Liver of rat fed dimethylaminobenzene-1-azo-1-naphthalene (DAN). Note proliferation of small, poorly developed bile ducts in portal areas, extending into adjacent lobules and sometimes isolating hepatic parenchymal cells. In *lower left* corner there is a group of smaller parenchymal cells representing a focus of periportal hyperplasia. Hematoxylin and eosin. × 135

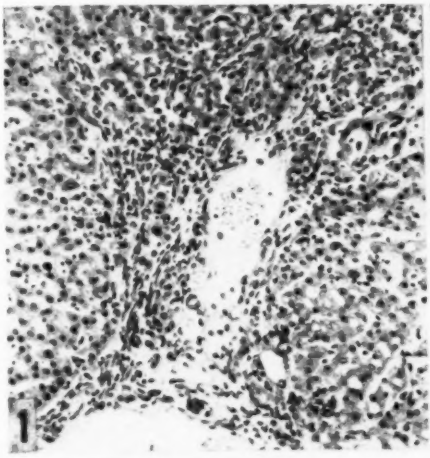
FIGURE 2.—Liver of rat fed diacetylaminofluorene (diAAF). Note proliferation and ectasia of larger bile ducts with formation of multiloculated cysts lined by flattened endothelial-like cells. Hematoxylin and eosin. × 115

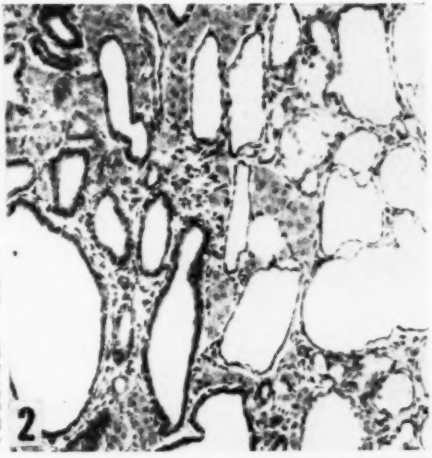
FIGURE 3.—Cholangiocarcinoma apparently arising in the center of a large multiloculated cyst at the margin of one lobe of the liver in a rat fed acetylaminofluorene. Hematoxylin and eosin. × 18

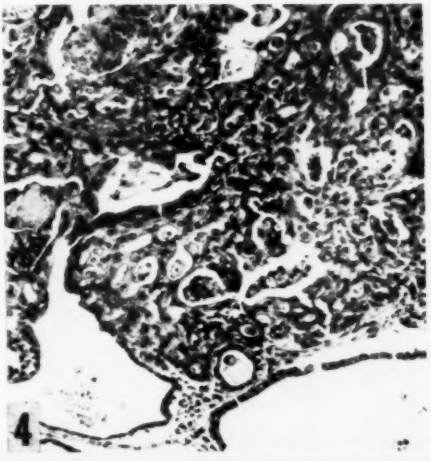
FIGURE 4.—Higher power of area marked on figure 3. × 120

FIGURE 5.—Bile-duct adenoma or cholangioma consisting of rather disorderly proliferation of larger bile ducts without cytological atypicality arising in the liver of a rat fed DAN. Hematoxylin and eosin. × 35

FIGURE 6.—Area of cholangiofibrosis in liver of a rat fed DAN. Hematoxylin and eosin. × 75







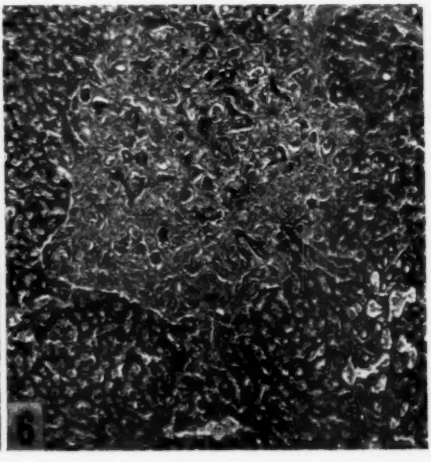


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PLATE 99

FIGURE 7.—Cholangiocarcinoma showing typical ductal and acinar pattern with periodic acid-Schiff positive mucus (shown in black) free in lumina and as large single spheres in the cytoplasm of goblet-type cells. Tumor occurred in rat fed DAN. Periodic acid-Schiff stain. × 160

FIGURE 8.—Hyperplastic focus of hepatic parenchymal cells in periportal area of the liver in a rat fed diAAF. Hematoxylin and eosin. × 125

FIGURE 9.—Hyperplastic nodule of parenchymal cells with obscure sinusoids and poorly defined cords distorting adjacent liver cords. Liver of rat fed diAAF. Hematoxylin and eosin. × 125

FIGURE 10.—Morphologically early hepatoma found in liver of adult male A × C rat 9 weeks after beginning continuous feeding of diAAF in diet. Grossly this nodule measured only 3 mm. in diameter. Insert shows low-power view × 18. The remainder of the picture shows variability and atypicality of the cells, absence of stroma, loss of cohesiveness of cells (partially post-mortem artefact) and areas of degeneration and necrosis. Hematoxylin and eosin. × 120

FIGURE 11.—"Trabecular hepatoma" in liver of rat fed DAN. Note double cords of liver cells in lower portion of picture; this is typical of embryonic rather than adult pattern. Hematoxylin and eosin. × 120

FIGURE 12.—"Adenohepatoma" composed of neoplastic parenchymal cells arranged around spaces not lined by endothelial cells and containing membranous debris and occasional degenerating cells. These spaces would appear to correspond to dilated bile canaliculi. Hematoxylin and eosin. × 120

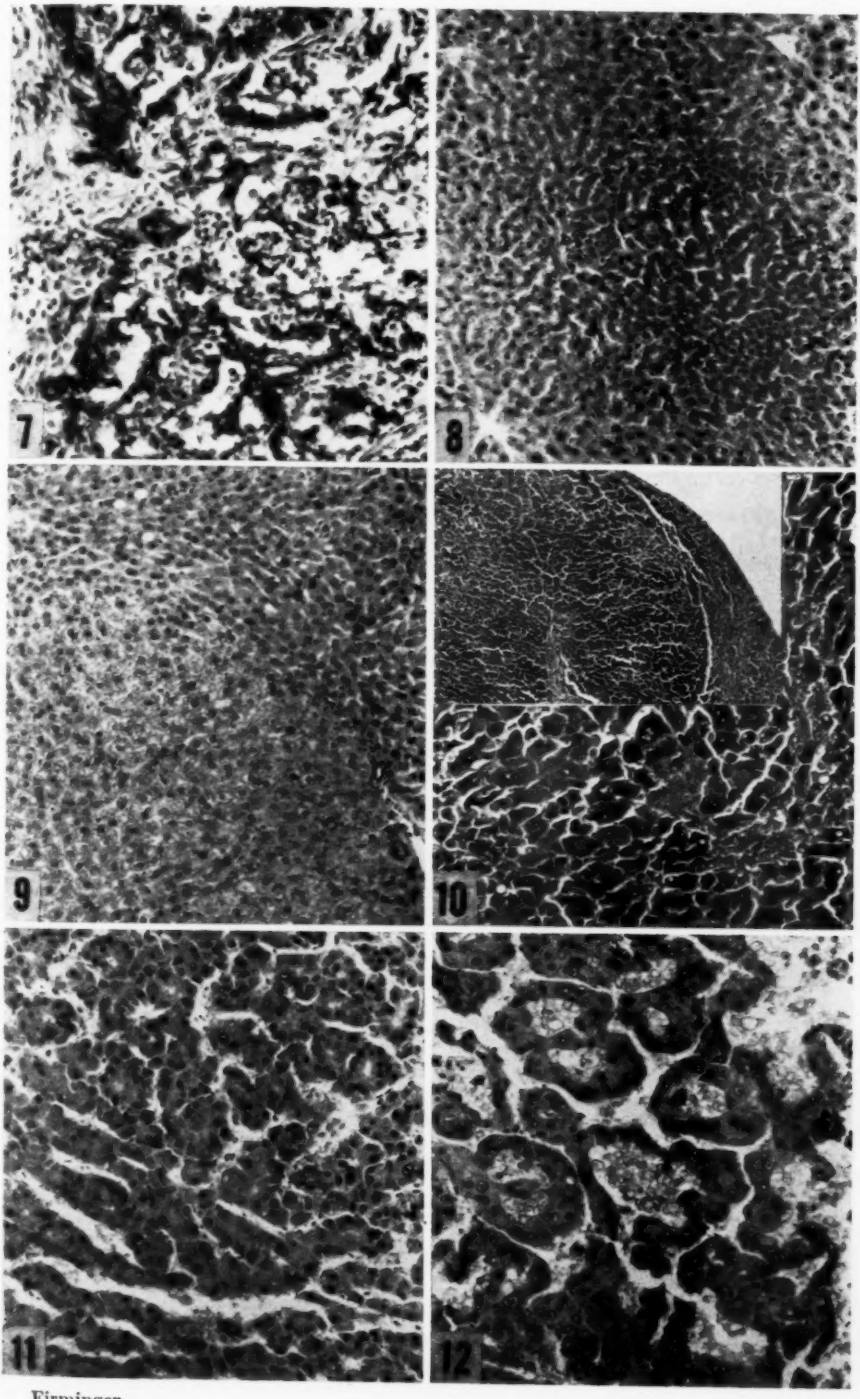
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PLATE 100

FIGURE 13.—Two varieties of hepatic cells in hepatoma in rat fed DAN. Hematoxylin and eosin. × 115

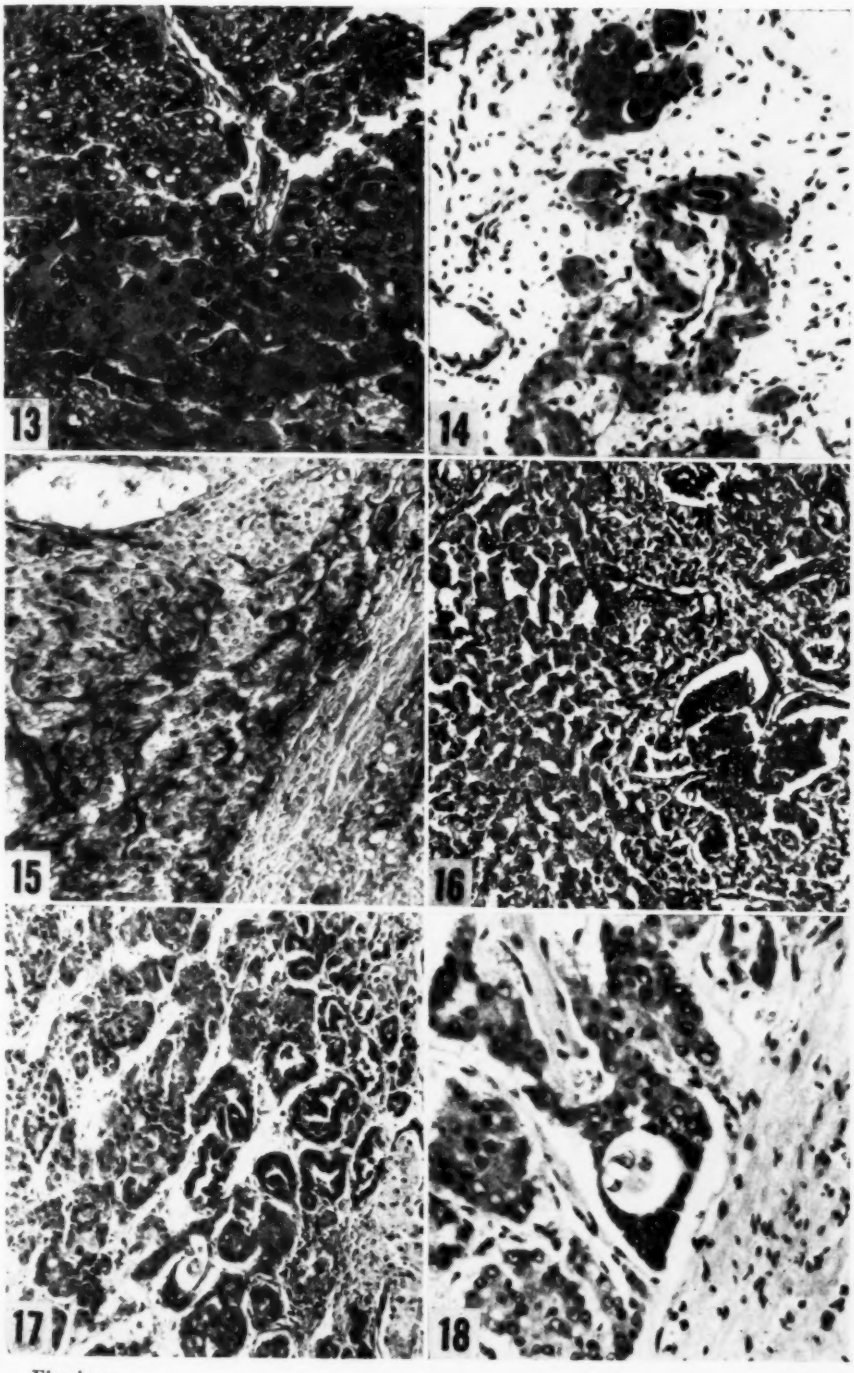
FIGURE 14.—Well-differentiated liver cells in metastatic teratoma of testis in a supraclavicular lymph node of human patient. Note plugs of bile pigment in canaliculi. This resembles findings often observed in hepatomas in human beings. Hematoxylin and eosin. × 180

FIGURE 15.—Alkaline phosphatase technique on hepatoma in liver of rat fed DAN. Note localization in sinusoidal endothelial cells and paucity of reaction in cytoplasm of tumor cells. × 120

FIGURE 16.—Cholangiohepatoma in the liver of a rat fed butter yellow. Note parenchymal cells comprising hepatomatous portion on the *left* and bile-duct type of epithelium with clearer cytoplasm on the *right*. Hematoxylin and eosin. × 90

FIGURE 17.—Cholangiohepatoma in the liver of a rat fed DAN. Note hepatomatous pattern on the *left* and cholangiocarcinomatous pattern on the *right*. Hematoxylin and eosin. × 120

FIGURE 18.—Hepatoma in the liver of a rat fed DAN and showing what resembles the formation of a bile duct at one point, yet there was no cholangiocarcinoma in this area. Hematoxylin and eosin. × 290



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Dr. E. Shelton, National Cancer Institute, Bethesda, Md.: As Dr. Firminger has so clearly illustrated, tumors induced in the liver of rats by azo dyes are complicated and diverse in structure and etiology. I would like to report briefly on some further work with the azo-dye induction of liver tumors in mice. The mouse hepatoma has the advantage of being derived from only one type of cell, the hepatic parenchymal cell. This is an advantage if one wishes to study the succession of events leading to the formation of a tumor. Last spring at the Cancer Meetings I reported that it was possible to obtain an incidence of about 80 percent of liver tumors in a period of 4 months by feeding *o*-aminoazotoluene to strain A female mice injected with a 10 percent stilbestrol-cholesterol pellet.

Since last spring further experiments have shown that liver tumors begin to appear in the mice as early as 28 days after the start of dye feeding. A 100-percent incidence of gross liver tumors may be produced in 90 days with only 70 days of dye feeding. In contrast to azo-dye carcinogenesis in the rat, high riboflavine (10 γ per gram) in the diet does not protect mice against the tumor-formation by *o*-aminoazotoluene. However, the strain of mouse used does seem to be important to the rapid production of a high percentage of liver tumors. An attempt was made to use a cross between two strain A sublines in order to obtain a more vigorous animal. It was found that a significantly lower number of tumors appeared in the hybrid, indicating that genetic factors play an important role in hepatic tumor production in the mouse.

Details of these experiments will be published at an early date.

Some Recent Findings Concerning Hepatomas Induced with *p*-Dimethylaminoazobenzene[1]

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While listening to reviews and discussions, I recall what I knew and thought in the early 1930's when the words "experimental hepatoma" were first introduced. Progress and expansion in knowledge on the subject is tremendous and striking, owing to American scientists, many of whom are present here. Faithfully responding to a kind invitation to take part in the discussion, I would like to state some of my views and also to add our latest findings.

The primary changes, caused in the rat liver by butter yellow, *p*-dimethylaminoazobenzene (DAB), are central atrophic degeneration and peripheral serous hepatitis (Roessle). The comparatively normal liver cells react with hypertrophy and increase in nuclear volume and number. These changes are not specific to DAB because other noncarcinogenic compounds also cause similar changes, possibly more intensive and extensive. These changes are not essential for hepatocarcinogenesis, since they are hardly detectable in the experiment with small doses of DAB, still resulting in cancer development.

The change, slightly delayed in its appearance, is proliferation of the epithelial cells of the intralobular bile ducts. The proliferating cells can be distinguished as such by their cytologic characteristics and their location between the bile ducts and the bile capillaries. This is clearly demonstrated by injecting India ink from the extrahepatic bile-duct system. The epithelial proliferation is not proportional in degree and extent to the primary degenerative and inflammatory changes. Similar changes caused by noncarcinogenic compounds are not always followed by the epithelial proliferation. As the epithelial proliferation progresses, an adenomatous pattern is formed. Increase in the interstitial elements presents a fibroadenomatous structure. Widening of the lumens produces a cysto-adenomatous picture. The stroma tends to regress, instead of growing progressively and invasively. All these tissues are not transplantable. The epithelial cells, although often atypical in appearance and arrangement, degenerate and desquamate. On the other hand, fibrosis progresses in the interstitium. This is accentuated by fibrous development in the periportal area and in the perilobular inflammatory zone, eventually leading to an annular type of cirrhosis, which can be accompanied by compensatory lobular and pseudolobular hyperplasia. Similar epithelial proliferation and consequences are demonstrated in rats by noncarcinogenic compounds such as *m*-toluene diamine, tetramethyldiaminobenzophenone, etc., and also in resistant species of animals such as rabbits by DAB. In fact, prolonged administration of small doses of purified DAB in rats can induce liver cancer without being complicated with the adenomatous bile-duct proliferation and cirrhotic changes.

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[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 11, 1954.

The primary foci of hepatocarcinogenesis are observed in unspecified areas, either in peripheral or interior regions of the lobules and even in the pseudolobules. Each focus is microscopic in size, consisting of a small number of cells. The cells are polygonal, smaller than normal liver cells, basophilic, and rich in chromatin substances. They are arranged in a solid strand, sprouting from a normal liver-cell strand and surrounded by sinusoids. The proximally located cells are transitional to the normal liver cells. The focus with a larger number of cells presents twisted and branched strands. The cells at distal parts of the strands often appear different, being larger, more basophilic, and with hyperchromatic nuclei and large nucleoli. Mitoses are frequent. These cells are arranged somewhat in trabecular and rosette formations. The cell groupings are not always completely bordered by fibril elements. The area of these atypical changes is clearly distinguished from the neighboring normal liver tissue. This is the smallest recognizable tissue in which histologic characteristics are identical to those found in progressively growing, metastasizing, and transplantable liver cancer. Quantitative increase of the atypical cells gradually forms an atypical hyperplastic nodule and eventually a macroscopic tumor.

Histologic structures of the tumor vary to some extent, depending upon forms of cell grouping and development of the sinusoids and interstitial elements. They can be lobular, alveolar, trabecular, adenomatous, and papillary. Mixed tumors with two or more types and transitional tumors between these types are also observed. Metaplasia can occur as in other carcinogenesis. A columnar cell type is frequently formed. Mucoid-producing, spindle, squamous, syncytial, and hypernephroma-like cell types, which have been reported in human cases, are also noted. A reticulum-cell tumor, seldom observed, however, is to be excluded from this category. If the columnar tumor cells are arranged in a papillary structure, this part of tissue appears cholangioma-like. Cholangioma, true in nature and sense, has never been induced by DAB. The extrahepatic bile ducts are always free from a tumorous change.

The atypical hyperplastic nodules develop multicentrically. After prolonged experiments, various stages of tumor development are observed, ranging from the primary foci to the macroscopic tumor. A wide and careful examination of many sections from one liver often reveals regions of different tumor types. Even one microscopic field sometimes shows mixed types, usually presenting one or the other type dominantly. Tumor types which develop after transplantation depend on tumor types involved in the transplant. One type, however, can happen to be found growing, the other being screened or masked. A metastatic tumor usually contains one type. Tumors which grow by its transplantation are of the same one type through many generations of passages.

The induced liver tumor is prone to invade the peritoneum, often causing peritonitis carcinomatosum. Small tumor masses can be found suspended in ascitic fluid. The tumor cells have a strong affinity to remain attached to their own masses. Even the daughter cells produced from the tumor cells on the surface do not loosen freely in ascitic fluid, but mingle among other cells. Tanaka demonstrated this process by microcinematography. Consequently, free tumor cells are scarcely found in ascitic fluid, and thus the chance of obtaining a free-cell ascites tumor by the transplantation of ascitic fluid is slight. Tanaka injected ascitic fluid of peritonitis carcinomatosum from 42 rats with induced liver cancer intraperitoneally into fresh rats. The specimen in only one rat produced ascites tumor characterized by free tumor cells. The ascites tumor obtained is transplantable through passages as such. Each of the free tumor cells produces free daughter cells instead of organizing a cell mass. This was demonstrated by microcinematography. These observations suggest that some tumor cells accidentally alter their constitutional property which is characteristic of epithelial cells. Tanaka succeeded in causing the same change experimentally by treating pieces of liver tumor tissue with thiourea or trypsin prior to transplantation. Free ascites tumors were constantly obtained which have been maintained through generations of passages. It is significant that such a simple experimental procedure may cause a permanent change in a cell property.

It has been previously noted, that distribution of the normal liver cells with various volumes of nuclei follows the Jacobi principle, while the liver-tumor cells show a higher peak at a larger nuclear volume and, in addition, extraordinary peaks, not ruled by the principle. As expected from this finding, chromosome numbers of the tumor cells vary widely. The cells are aneuploid. Hyperdiploid chromosome numbers are especially frequent, showing a peak at 53. Recently, Ohno has developed a technique by which chromosomes in interphase cells are distinctly demonstrated. Two types of interphase chromosomes are distinguished: A) double-stranded, long, and uncoiled chromosomes, similar to those at early prophase, and B) double-stranded, short, and coiled, similar to those at telophase before nuclear reconstruction. For instance, the rat myeloblasts which can reproduce themselves by mitosis have the A chromosomes, while the rat leukocytes and normoblasts which are at the terminal phase of life or do not repeat mitosis have the B chromosomes. The myeloblast at early prophase has double-stranded, long, and uncoiled chromosomes, directly transitional in form to the A type. They become short and coiled between late prophase and early metaphase. At late metaphase, they split to single-stranded chromosomes which are reduplicated between late anaphase and early telophase, forming double-stranded, short, and coiled chromosomes, directly transitional to the B type. During nuclear reconstruction at late telophase, however, the chromosomes again become long and uncoiled, forming the A-type interphase chromosomes. This is a complete interphase-mitosis-interphase cycle. Besides all myeloblasts, most promyelocytes and some pronormoblasts and myelocytes can reproduce themselves by repeating the cycle. However, most myelocytes and some pronormoblasts show a different process. The telophasic chromosomes remain short and uncoiled through the stage of nuclear reconstruction and form the B-type interphase chromosomes. The dividing daughter cells in this case appear as the leukocytes and normoblasts. This is the terminal mitosis. The cycle is no longer complete. The cells in the terminal mitosis are usually hypoploid. Loss of certain chromosomes may be responsible for the process of the B type. Hypoploidy is frequent in relatively matured cells. The interphase chromosomes are less than diploid in the leukocytes and normoblasts. The normal and malignant liver cells were similarly studied. The interphase chromosomes of most liver-tumor cells belong to the A type and those of the normal liver cells are of the B type. Anticancer compounds, for instance TEM, cause dislocation of chromosomes besides other cytologic injuries. This leads to reduction of chromosomes. These studies, although they are still at an immature stage, appear to have brought out new problems. At least, some interpretations are given on interphase nuclei beyond prevailing descriptions, such as rich or poor and fine or coarse chromatin substances.

Quantitative Histology of Rat Liver During Carcinogenesis by *p*-Dimethylaminoazobenzene[1, 2]

ROGER DAOUST,[3] *Research Laboratories, Montreal Cancer Institute, Notre-Dame Hospital, Montreal, Canada*

Numerous biochemical studies have been carried out in this and other laboratories on homogenates of livers of rats fed *p*-dimethylaminoazobenzene (DAB) and liver tumors induced with DAB. For many years, chemical data have been expressed in terms of units such as wet weight, dry weight or tissue nitrogen. Recently, however, a few investigators started to estimate the number of nuclei present in the homogenates and to express the biochemical data as amounts or activities "per cell." This method of referring to the *average cell* has indeed greatly facilitated the interpretation of results in terms of the structural units of tissues.

Most tissues are composed of several cell types and, consequently, the *average cell* seldom represents a single cell type. It appears, nevertheless, as a reliable criterion for studying tissue in various physiologic or pathologic conditions, as long as the proportion of the different cell types remains constant. In rats fed DAB, however, the cytologic composition of the liver is greatly altered during the progress of carcinogenesis (*1–4*) and we felt that the expression *average cell* might have quite different meanings at various phases of carcinogenesis. In order to evaluate these changes and to define the *average cell* in these conditions, we have undertaken to measure quantitatively the proportion of the various cell types present in the liver at different time intervals during DAB feeding.

Adult male albino rats were fed a semisynthetic diet that contained 0.06 percent of DAB. Groups of animals were sacrificed at 30-day intervals. Pieces of the median and left lateral lobes of the livers were fixed in Susa fluid. Histologic sections were stained with hematoxylin and eosin. Differential counts of parenchymal cells (P), littoral cells (L), cells of bile ducts (B), cells of connective tissue (C) and cells of blood vessel walls (V) were made on these sections under oil immersion. Six thousand nuclei or more were examined per animal.

This quantitative analysis has revealed that the changes which take place in the cytologic composition of liver during DAB carcinogenesis (text-fig. 1) are much more important than one might suspect from simple microscopic observation.

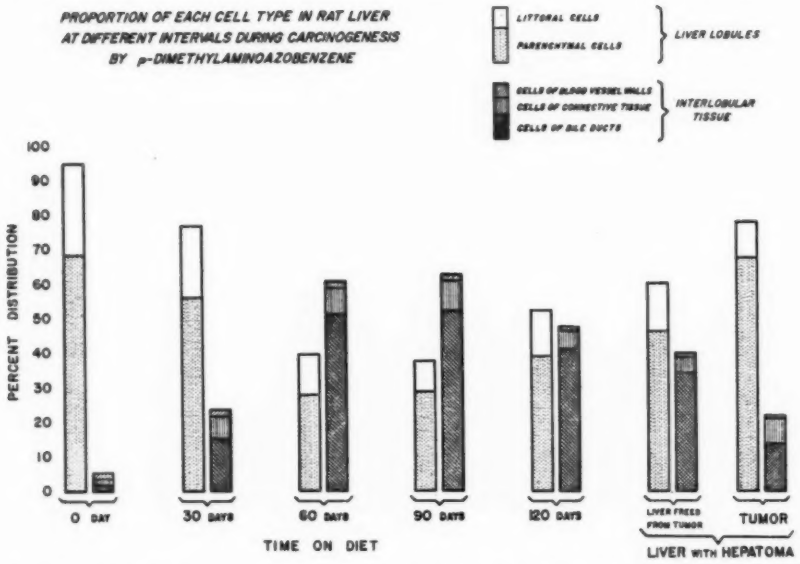
In normal animals, cells of liver lobules make up 95 percent of the total number of liver cells (P: 68%; L: 27%), while the interlobular tissue forms only 5 percent (B: 2%; C: 2%; V: 1%).

During the initial stages of DAB feeding, interlobular tissue infiltrates the liver and, consequently, the percentage of cells composing the lobules progressively decreases and reaches a minimum after 90 days. At this time, parenchymal and littoral cells account for only 29 percent and 9 percent, respectively, of the total number of cells,

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 11, 1954.

[2] This investigation was supported by a grant from the National Cancer Institute of Canada to Dr. A. Cantero, Director of the Research Laboratories.

[3] Fellow of the Damon Runyon Memorial Fund for Cancer Research.



TEXT-FIGURE 1.—Changes in the cell population in rat liver during carcinogenesis.

while the interlobular elements compose 62 percent (B: 52%; C: 9%; V: 1%) of the liver-cell population. After this period, due to a regression of interlobular tissue, the percentage of parenchymal and littoral cells increases, but remains below normal value. The livers freed from tumors (150 or 180 days of DAB feeding) were composed of 47 percent of parenchymal cells, 13 percent of littoral cells and 40 percent of interlobular cells (B: 34%; C: 5%; V: 1%).

The types of tumors induced by DAB were either pure hepatomas or mixed hepatoma (tumor of parenchymal cells) and cholangioma (tumor of bile-duct cells). In the so-called "pure hepatomas," the percentage of parenchymal cells was the same as in normal liver, namely 68 percent; the percentage of littoral cells, 10 percent, was lower than normal, and interlobular elements formed 22 percent of the tumor cells (B: 14%; C: 8%; V: $<1\%$). In tumors of the mixed type, large variations were observed between individuals in the relative proportions of parenchymal and bile-duct cells. In some cases, bile-duct cells were present in relatively low number, while in others they were in great majority.

Correction of our data for the nuclear sizes of the various cell types will somewhat lower the percentage of parenchymal cells at each period. (The nuclei of these cells being larger than those of the other cell types, they have higher chances to be present in any section.) The present data clearly show, however, the extent of the changes which take place in the liver-cell population during DAB carcinogenesis.

Thus, our results indicate that the cytologic composition of rat liver is extensively altered during DAB feeding, particularly at the 60- and 90-day intervals. Since cells of various types usually differ in chemical composition, changes in the proportions of various cell types are likely to alter the average chemical composition of a tissue. Therefore, variations in composition of the *average cell*, as determined in tissue homogenates, do not necessarily mean real variations in individual cells; they may simply reflect variations in the cytologic composition of the tissue, and must be interpreted with caution. It should be considered, for instance, that the *average cell* which, in normal liver, is ⅔ a parenchymal cell and ⅓ a littoral cell, has become, after 90 days of DAB feeding, close to ⅔ a bile-duct cell and less than ⅓ a parenchymal cell. On

this basis alone, it should not be surprising to find that the *average cell* has a different chemical composition at the 90-day period, as compared to normal.

Stowell (*5*) and other authors have stressed the need of a "cytochemical viewpoint" in studies of tissue homogenates. In the recent years, an important step has been made in that direction by expressing biochemical data on a "per cell" basis. The present study demonstrates that, in addition, the cytologic composition of the investigated material must be defined as precisely as possible in order to attempt reliable interpretation of the biochemical data.

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Human Hepatocarcinoma and the Comparative Embryology of the Vertebrate Liver[1, 2]

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Two years ago Dr. Hans Popper suggested that I undertake a study of the three-dimensional morphology and histogenesis of primary carcinoma of the human liver. Since relations between embryology and carcinogenesis are assumed to exist, it was felt necessary to reinvestigate the embryology of the human liver; for the accounts on the subject hitherto presented appeared unsatisfactory (*1*), especially since the basic concept of liver structure had been changed from the cord theory (*2*) to the plate theory (*3*).

When, as a preparatory exercise to the understanding of hepatocarcinogenesis, the embryology of the liver of man was studied, a most astonishing phenomenon was observed; namely, that mesoderm (and especially the visceral mesothelium) makes a considerable contribution to the hepatic parenchyma. This surprising fact seemed, in itself, hard to understand, and for a better perspective an extended study of the origin and early development of the livers of 25 species, distributed among all classes of vertebrates, was undertaken. A preliminary account and a complete monograph of this comparative-embryologic study are going to press (*4*, *5*). It will be seen that the results of this comparative-embryologic survey can shed light on the understanding of the morphology and development of human hepatocarcinoma.

Embryologic Observations

Among 25 species investigated by the author, to which are added 5 species previously observed by other authors, 12 basically different types of hepatogenesis (*i.e.*, development of liver parenchyma) exist. Superimposed on them are 2 basic types of cholangiogenesis (*i.e.*, development of the bile ducts). In the following lines only a few strikingly characteristic modes of liver development can be sketched:

A. Hepatogenesis

1) Tubules.—In certain vertebrates, such as in two species of sharks, the electric ray, the alligator, and the chick, the early liver anlage consists of sprouting tubules (text-fig. 1,*B*; figs. 1 and 2). In the cases of the shark *Scyllium* and the chick, these tubules are purely entodermal; but in the shark *Squalus*, the electric ray, and the alligator, the visceral mesothelium makes a substantial contribution to the mass of primitive liver tubules. In figure 1, the mesodermal liver tubules seen dorsolaterally, particularly on the left side, are still separated from the entodermal part of the liver. Whether the tubular liver anlage is purely entodermal or a mixture of the inner and middle germ-layer, its further development continues along the same lines. Neigh-

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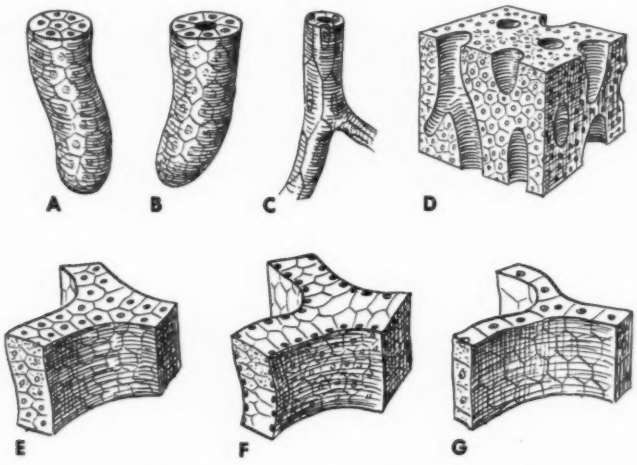
boring tubules come to touch each other and fuse, forming plates of two-cell thickness (fig. 2) as is characteristic of the adult livers of these forms.



TEXT-FIGURE 1.—Schematic stereograms to show various structural types of embryonic and adult livers as well as of carcinomatous structures.

A. Solid cords (pig embryo; carcinoma).

B. Tubule (embryos of lampreys, sharks, rays, snakes, alligators, birds; some carcinomata).

C. Cholangiole.

D. Muralium composed of walls several cells thick (embryos of amphibians, turtles and mammals; some carcinomata).

E. Walls two cells thick without nuclear polarity (adult bony fishes, amphibians, certain turtles and lizards, some lower birds; some cirrhotic nodules; some carcinomata).

F. Walls two cells thick with nuclear polarity (older embryos of sharks, snakes, turtles, lizards, alligators, certain lower birds; some cirrhotic nodules, some carcinomata).

G. Walls or plates one cell thick (normal liver of adult mammals and song birds).

2) Cavitation of solid cell masses.—In the interior of large masses of cells, blood islands form. These fuse and become the liver sinusoids. In amphibians, this process occurs in the anterior portion of the entodermal yolk cell mass. The blood vessels which arise in this mass are at once vitelline veins and liver sinusoids. In the turtle, it is a mass of cells constituting a massive, multilayered mesothelium (consisting partly of visceral peritoneum and partly of parietal pleura) with a few entodermal cells similarly cavitated (figs. 3 and 4). These masses form walls several cells thick between the sinusoids, and are thinned down during early development to plates two cells thick (turtle) or to plates one and two cells thick (frog).

3) Interstitial invasion.—This process, observed simultaneously by Lipp (*1*) and by the writer, is as follows: Individual cells and cell groups become detached from a vigorously proliferating epithelial layer. They invade the interstitial mesenchyme present in the meshes of pre-existing capillary networks. These capillary networks are connected with the omphalomesenteric and umbilical veins.

In the guinea pig (fig. 5), interstitial invasion occurs from the thick-walled, entodermal liver diverticulum alone. The invading cells surround the sinusoids and form the primitive liver plates (fig. 5). In the opossum, the prosimian *Microcebus*, the rat and man, interstitial invasion occurs from both the entodermal liver diverticulum *and*

from the *mesodermal* visceral mesothelium. Entodermal and mesodermal cells intermingle in the intercapillary spaces and together form the primitive liver plates (fig. 6).

The primitive liver plates (*i.e.*, intercapillary liver-cell masses) are at first several cells thick and later flatten out to a thickness of one cell.

4) Mesenchymal transformation together with entodermal trabeculation.—In the pig, entodermal trabeculae or cords penetrate into intercapillary spaces and then broaden to form plates several cells thick which surround these vessels. This process occurs cranially and laterally from the liver diverticulum. Caudally, mesenchymal cells present in the meshes of an omphalomesentero-umbilical capillary plexus are directly transformed into liver cells by swelling of the cytoplasm and by its becoming strongly acidophilic and by transformation of the nuclei.

These seven modes of hepatogenesis will suffice as a sample. The reader interested in more detail may obtain further information in two papers to be published shortly (*4*, *5*).

B. Cholangiogenesis

In some vertebrates, the bile- and hepatic-duct systems are direct prolongations of the gut (shark, ray, bow fin, garter snake, alligator, chick, song birds). In other vertebrates, the ducts originate peripherally from liver cells and grow from the liver toward the intestine, joining it secondarily (lamprey, guppy, salamander, frog, *man*).

C. Adult Liver Structure

In spite of this astonishing variety of developmental patterns, the end result of development is remarkably uniform: The liver of every investigated vertebrate is a continuous mass of cells, recently named a "muralium" (system of walls) (*7*). This mass is tunneled by the *lacunae hepatis* which, together, form the *labyrinthus hepatis*. The continuous network of sinusoids is suspended in this labyrinth (*8*). Basically the livers of all adult vertebrates,[1] then, are identical in structure. There is but one difference: In the lower vertebrates the liver walls are predominantly two cells thick, while in song birds (*9*) and in mammals (*3*) the plates are uniformly one cell thick. The plate thickness in amphibians varies between a thickness of one and two cells in the same muralium.

Morphologic Liver Types in Vertebrate Embryos and Adults

Reviewing the structure of the liver of various vertebrate embryos and adults, we may be able to arrive at a morphologic classification of these structures which might be helpful in the understanding of human hepatocarcinoma.

Solid cords such as are shown schematically in text-figure 1,*A* have been found in the entodermal portion of the early liver primordium of the pig.

Typical tubules (text-fig. 1,*B*) are encountered in embryos of lampreys, sharks, rays, alligators, garter snakes, chicks and in older lamprey larvae during metamorphosis.

The muralium is a continuous cell mass tunneled by lacunae which contain sinusoids. Four types of muralia can be distinguished. The difference between them lies in the histologic thickness of the partitions (walls, plates, *laminae hepatis*) that separate neighboring lacunae from one another.

Muralia consisting of walls with a thickness of three or more cells (text-fig. 1,*D*) exist in embryos of salamanders, frogs, turtles, and all mammals studied, including, of course, man.

There are two types of muralia with walls of two-cell thickness; namely, those with or without nuclear polarity. In a muralium of plates of two-cell thickness without nuclear polarity, the nuclei of the hepatic cells lie approximately in the middle of the cells (text-fig. 1,*E*). This type is encountered in adult bony fishes, such as the carp and the moray; in amphibians, *e.g.*, *Necturus maculosus;* in a reptile, such as the turtle, *Clemmys insculpata*, and in the European lizard, *Lacerta agilis*.

[1] Except for the adult lampreys in which a regressive development occurs during metamorphosis (*6*).

Muralia consisting of laminae of two-cell thickness with nuclear polarity are characterized by an eccentric location of the liver-cell nuclei. The nuclei lie in the basal part of the cell, that is, nearest the blood stream, and near the surface of the liver plates. This type is illustrated in text-figure 1,*F*. It is encountered in the embryos of sharks, turtles, garter snakes, and in some reptiles, such as the adult lizard, *Eumeces absoletus*, and the alligator; also in the chicken and the wood duck.

Amphibians (mudpuppies, salamanders, tree toads, frogs) have muralia of mixed walls, namely, walls which are one and two cells thick (not illustrated).

Finally, we must mention the muralium of liver plates one cell thick which is found in some lower and in all the higher birds (*Passeriformes*=song birds) as well as in all mammals, beginning with such low forms as the spiny anteater, up to man. This type is illustrated in text-figure 1,*G*. It is mechanically the most stable and physiologically the most efficient type of liver structure (*10*).

All these types, as well as other structures, can be found in human hepatocarcinomata.

A Preliminary Survey of Human Hepatocarcinomata

So far, 61 cases of primary liver carcinoma from the Department of Pathology at Cook County Hospital and 38 selected cases from the files of the Armed Forces Institute of Pathology have been submitted to a preliminary survey of morphogenetic potentialities. The author is most grateful to Dr. Hans Popper and to Captain William M. Silliphant for this material. He is aware of the preliminary nature of this survey, for the total number of 99 cases examined to date is quite insufficient to arrive at an understanding of so versatile a phenomenon as hepatocarcinoma. Also, three-dimensional reconstructions of these tumors have not yet been made.

While the purpose of this paper is to correlate the hepatocarcinoma with comparative-embryologic findings, a few additional phenomena were observed which have no immediate connection with normal vertebrate embryology. For the sake of completeness, all observations hitherto made will be mentioned; among them, there is much confirmation of well-known phenomena which have been so masterfully synthesized by Edmondson and Steiner (*11*).

1) Direct transformation of liver cells into carcinoma cells.—As was so strikingly illustrated at the recent symposium of the International Congress of Pathology (*12*), pathologists do not yet agree on whether a carcinoma originates from one single cell which multiplies or whether it is polyphyletic. To this problem special attention was given when surveying 45 cases of tumors at the Armed Forces Institute of Pathology. Of these 45 cases, only 38 were included in the study; namely, those cases in which direct transitions of normal or cirrhotic liver cells into tumor cells could be ascertained.

Figure 7 shows such direct transformation. It is silver impregnated by Wilder's method to show argyrophil fibers, nuclei and cytoplasm. At the right, normal liver-cell plates one cell thick are ensheathed in reticular tissue. These plates are continuous with thicker plates which have lost their acidophilia and which have acquired basophilia as can be seen in the adjacent section stained with thionin and eosin (not illustrated). They have increased in histologic thickness, having become two and more cells thick. These enlarged, cancerous cell masses are obviously transformed liver plates, as can be seen by the fact that the reticular lining of the normal liver plates is continuous with the lining of these new, cancerous cell plates and masses. An entire cancerous cell group is contained in the original argyrophil sheath of the original liver plate. The argyrophil sheath is thinned out since it has been stretched by the growth of the neoplastic cell mass contained in it. No argyrophil fibers can be found within these newly formed cancer plates. All these observations clearly show that what is contained between the thinned out argyrophil layers must have been, not long ago, a normal liver-cell plate, the cells of which have abnormally multiplied.

In many cases (figs. 8 and 9) liver-cell plates exposed to pressure become transformed into tumor cells.

Much evidence suggests that pressure, plus the presence of carcinogenetic substances, is often instrumental in bringing about the transformation. Transformation can be seen simultaneously at many places in the same liver.

The transformation into carcinoma cells can occur from normal liver cells or from cirrhotic blastema, as described by Popper and Elias (*13*).

It is commonly believed that normal liver cells do not change into tumor cells. Pictures showing this transformation are interpreted as invasion by tumor cells of intersinusoidal spaces, in other words as replacement of liver plates by tumor plates. If this were true, debris left behind by necrotic cells and evidence of pressure perpendicular to the alleged direction of invasion should be encountered. One also is at a loss to explain the gradual transitions between normal-appearing liver plates and the adjacent tumor-cell masses. If the invasion theory were correct, the tumor cell nearest to a normal liver-cell plate should be of a highly advanced carcinomatous appearance. Also the continuity of the smooth outlines of entire liver plates, which on one side contain normal liver cells and on the other cancer cells, cannot be understood on the basis of the invasion theory. And, in the face of absence of necrosis at the boundary, one should expect at least to find fragments of healthy liver cells phagocytosed by the supposed invaders. None of these logical consequences of the invasion theory is verified. A good demonstration of direct transformation is seen in figures 8 and 9: Here a small territory of liver is expanding. As it expands it exerts pressure upon adjacent liver-cell plates. The cells belonging to this tract of liver plates undergo a distinct change toward a carcinomatous character; namely, a considerable increase in size, an increase of cytoplasmic basophilia, and the nuclei become large and hyperchromatic. This change is occurring in a large territory of apparently normal liver far away from the nearest tumor.

2) *Transformation of liver-cell plates into cholangioles.*—At the periphery of a growing nodule, which appears still as a cirrhotic nodule, the blastema may be transformed into cholangioles (fig. 10). This is not astonishing, since the cholangioles arise normally from liver cells (*4*, *5*, *14*, *15*). Frequently, these cholangiole-like structures do not develop further but die. But in other cases they may become cancerous.

3) *Transformation of cholangioles into bile ducts.*—Where cholangioles are connected with a cancerous nodule, the cholangiole may swell into a large bubble acquiring a tall, columnar epithelium. It appears in some cases (fig. 11) as if a substance produced within the cancerous nodule were secreted into the cholangiole—thereby greatly dilating it. At the same time the pressure of dilatation plus the stimulus of the secretion may cause the epithelium of the cholangiole to undergo the change to tall columnar. Also, this phenomenon is embryologically understandable, since in *normal* development ducts arise from cholangioles (*5*, *15*).

4) *Direct transformation of normal liver plates into plates of two-cell thickness with polarity and from there into tubules.*—This transformation is frequently observed (fig. 12). Such tubules must not be confused with cholangioles, but in section they appear as typical glandular tubules, filled with bile, the cells lining them having the appearance of secretory cells. Text-figure 1,*C* attempts to demonstrate a cholangiole as distinct from a tubule (text-fig. 1,*B*).

Transformation into plates of two-cell thickness is regression to a lower vertebrate, adult type. The further transformation into tubules is a further reversion from the adult, lower vertebrate into a certain embryonic lower vertebrate type (chick, shark, snake, and alligator embryos). The regression from plates two cells thick into tubules is comparable to the regressive development normally found in lampreys (*6*). In these animals, plates two cells thick are first split into tubules. Then they undergo a further regression by the loss of their lumina and by splitting up into solid oval cell groups (*16*).

5) *Solid cords.*—These are well known in many cases of hepatocellular carcinoma. They may be compared with entodermal cords appearing early in the pig embryo (text-fig. 1,*A*). Three-dimensional reconstruction, undertaken while this paper was going to press, showed that such cords result from the splitting of thickened plates, a phenomenon exactly the reverse of normal liver development in the pig.

6) *Muralia of plates several cells thick.*—These appear in many human hepatomata. They may have great similarity to early liver primordia of turtles (figs. 3, 4, and 13).

7) *Infiltrating hepatoma.*—The infiltrating hepatoma is similar in structure and behavior to the earliest mammalian liver primordia (figs. 5 and 6) characterized by "interstitial invasion."

8) *Hepatosarcoma.*—It is generally known that a hepatoma may come to "resemble" a sarcoma. In three cases, a direct transformation of liver cells into spindle-shaped cells resembling fibroblasts was observed (fig. 14). Such a tumor, it may be suggested, does not just *resemble* a sarcoma. Perhaps it *is* a sarcoma. The fact that mesothelial cells and even mesenchymal cells are transformed into hepatic cells in normal embryos of so many species, including our own (see above), certainly gives us the right to suspect the possibility of transformation of liver cells into mesenchymal cells and fibroblasts.

9) *Transformation into nonhepatic structures.*—Such transformation has been known and was recently re-emphasized (*11*). However, the present preliminary survey has brought to light additional structures to my knowledge hitherto undescribed. This is only a matter of morphologic resemblance. Whether these structures also have functional resemblance to the organs which they mimic will be the object of future investigations.

a) *Thyroid-like follicles* have been known to occur in hepatoma. During the present observation three such cases were found. In one case, these follicles were filled with a colloid, peripherally vacuolated and having the mechanical and optical properties of true thyroid colloid, except that it also contained bile dyes, as seen from its brownish coloration (fig. 15). Direct transformation of normal liver-cell plates into thyroid-like follicles was observed (fig. 16). In this case, liver cells become rapidly and immediately dedifferentiated into primitive entoderm cells and then assume a structure reminiscent of another entodermal organ.

b) *Adrenoid transformation.*—One case of adrenoid transformation was observed. Normal liver-cell plates acted as a blastema (*17–19*) for what resembled cortical-cell groups. All three zones of the adrenal cortex could be distinguished in a few regions of this tumor. The most striking features are: *1*) direct transformation of normal liver plates which are exposed to pressure into zona glomerulosa-like tissue (fig. 17) and from there into zona fasciculata-like tissue, and then into zona reticularis-like tissue; *2*) the normal distribution of lipoid vacuoles is paralleled in this tumor. The adrenoid transformation can be explained by the embryologic observation that the mesothelium gives rise to a great part of the liver-cell plates. Therefore, it can be understood that liver plates, when dedifferentiating, again very suddenly, transform into a structure resembling another organ which normally arises from mesothelium.

c) *Ovaroid transformation.*—In four cases, numerous cancerous nodules were found, particularly among emboli in portal veins and in lung metastases which histologically resembled a corpus luteum of pregnancy (fig. 18). The similarity to corpus luteum was striking and was noted immediately.

In one of these four cases, *after* the diagnosis of corpus luteum-like structure had been made, a large cell containing a gigantic, spherical nucleus with an intensely red-staining, large nucleolus was found (fig. 19). The nucleus had the exact appearance of a large, almost mature oöcyte nucleus. One may compare it for size with the normal liver-cell nuclei near the bottom of the figure. After the discovery of this large egglike cell, numerous other such cells, but smaller, were found in other nodules in the same case, most of them in portal emboli; the large cell mentioned above, however, was found in an intraparenchymatous nodule. Small, oöcyte-like cells were also found in another case with corpus luteum-like nodules.

Again, after having learned that the mesothelium is an important contributor to the liver parenchyma, we are not surprised that the hepatoma can mimic an ovary. More surprising is the fact that these ovaroid structures were found in male patients.

It should be emphasized, however, that nothing has been seen here but mere *morphologic resemblance*. *No identity* is being claimed.

Summary

Twelve modes of development of liver parenchyma and two modes of ductal development are described for 30 species of vertebrates. Most astonishing among them are large mesodermal contributions to the hepatic parenchyma.

Direct transformations of normal and cirrhotic liver cells into carcinomatous cells are demonstrated in 38 of 45 cases. In 61 additional cases, no search was made for this phenomenon.

Transformation of liver cells into cholangioles and of cholangioles into large bile ducts is shown.

Various structural types found in human hepatoma are compared and correlated with similar structures found in adult lower vertebrates and in embryos of various vertebrates (lower vertebrates, mammalian and human).

Hepatosarcomata are explained on the basis of mesodermal contributions to the liver parenchyma.

Several nonhepatic structures which mimic other organs, such as thyroid, adrenal cortex and ovary, are demonstrated and their origin is explained on an embryologic basis.

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PLATE 101

FIGURE 1.—Section of shark embryo nineteen mm. long. Liver tubules are arising in two groups on each side; a ventral group of tubules arises as evaginations from the gut, a dorsal group of tubules from the mesothelium. × 150

FIGURE 2.—Section of shark embryo twenty-one mm. long. The tubules join forming walls two cells thick exhibiting nuclear polarity. × 150

FIGURE 3.—Section of turtle embryo. Young embryo showing the early liver anlage at the level of greatest entodermal participation. The liver anlage is a solid mass of cells presenting a mixture of entodermal and mesodermal elements. More anteriorly and more posteriorly, this mass is connected with the mesothelium only. Blood islands arise in this mass. They constitute the primitive liver sinusoids. × 80

FIGURE 4.—Section of turtle embryo. Slightly older embryo showing section of liver anlage. It is a muralium of plates several cells thick, very much resembling the carcinoma shown in figure 13. × 80

FIGURE 5.—Embryo of guinea pig. Masses of entodermal cells invade the spaces between the parts of a pre-existing capillary network. The capillaries are the primitive liver sinusoids; the advancing and invading cell masses surround them forming the primitive liver plates. × 100

FIGURE 6.—Human embryo of 27 pairs of somites (#7999 Carnegie collection). Cell masses from both the entodermal liver diverticulum and from the visceral mesothelium invade the intersinusoidal spaces forming the primitive liver-cell plates. × 100

PLATE 102

FIGURE 7.—Direct transformation of liver-cell plates into carcinoma. The normal liver-cell plates are seen at the *right*. They consist of acidophilic (dark-stained) cells. The cancer cells have lost the acidophilia (therefore they appear unstained). The reticular tissue surrounds the normal liver plates (*right*) and is continuous around the enlarged carcinoma masses (*left*). × 160

FIGURES 8 and 9.—High mechanical pressure on a tract of liver-cell plates influences their transformation into carcinoma. The figures show the same area under different magnifications. × 50; × 150

FIGURE 10.—Transformation of liver cells into cholangioles at the periphery of a cirrhotic nodule. × 90

FIGURE 11.—Transformation of cholangioles connected with a cancerous nodule into ducts with large lumen and high, columnar epithelium. × 90

FIGURE 12.—Transformation of plates two cells thick into tubules in a hepatocarcinoma. × 650

FIGURE 13.—Hepatocarcinoma showing the structure of a muralium of walls several cells thick very similar to that of the liver anlage of a turtle as seen in figure 4. × 150

PLATE 103

FIGURE 14.—Direct transformation of liver cells into spindle-shaped cells having the appearance of fibroblasts. × 550

FIGURE 15.—A hepatocellular carcinoma containing thyroid-like follicles. × 150

FIGURE 16.—Peripheral portion of the tumor shown in figure 15, showing direct transformation of liver-cell plates of normal structure into a thyroid-like follicle. × 550

FIGURE 17.—Portion of an intrahepatic, hepatocellular tumor having the structure of the adrenal cortex. At the *left* are some compressed, normal appearing liver plates. These act as a blastema for the formation of the adrenal cortex-like tissue. This photograph shows the zona glomerulosa with its characteristic high lipid content. Below it (not shown in the illustration) was a large zona fasciculata and below this a typical zona reticularis. × 550

FIGURE 18.—Intrahepatic nodule of a hepatocarcinoma having the texture of a corpus luteum of pregnancy. × 300

FIGURE 19.—A large oöcyte-like cell in an intrahepatic, hepatocellular carcinoma. × 500

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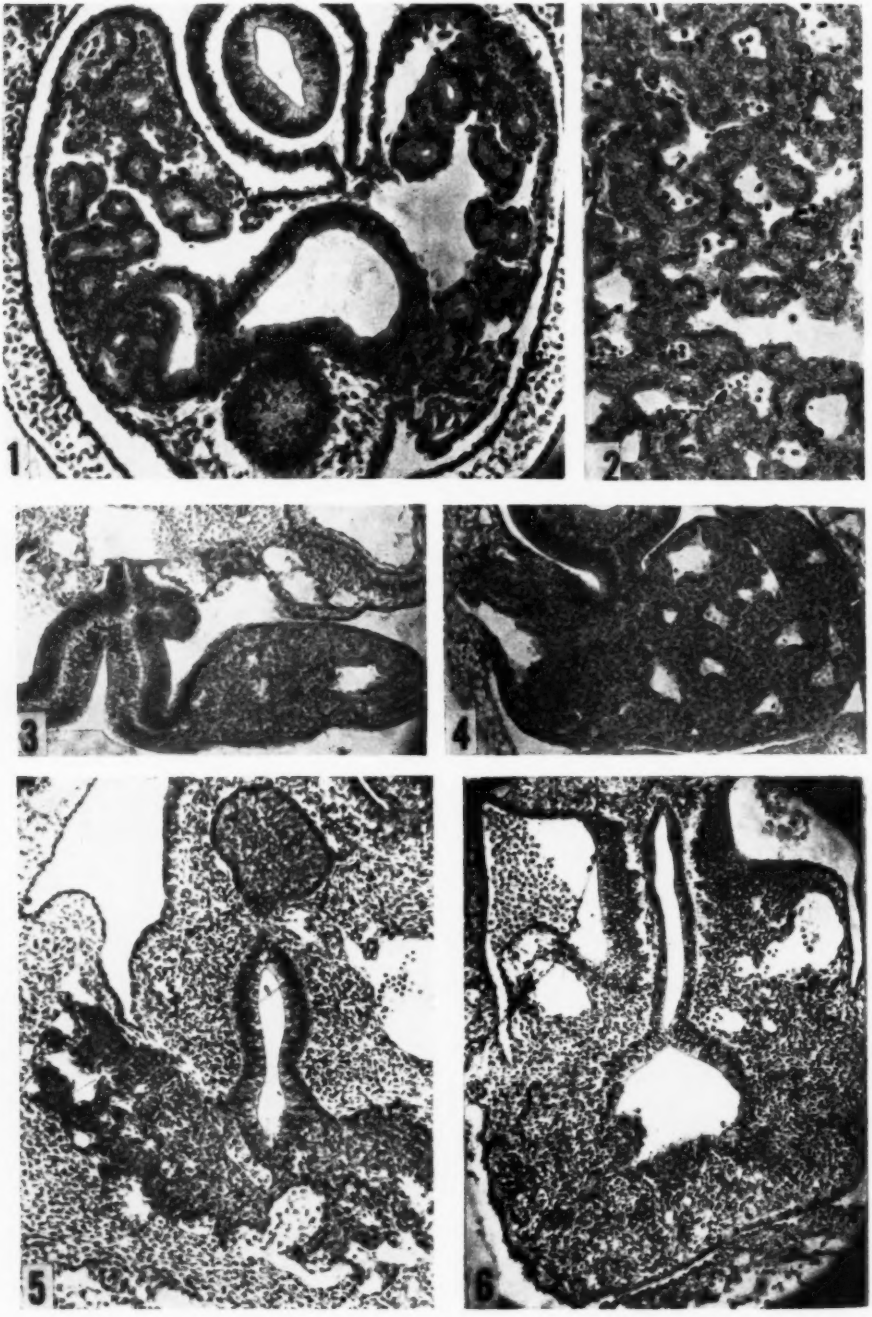
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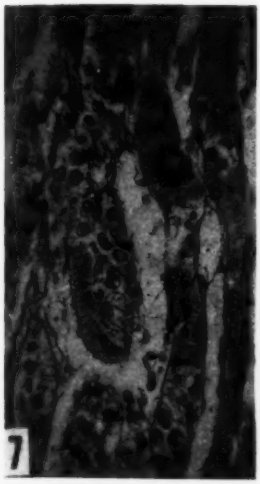
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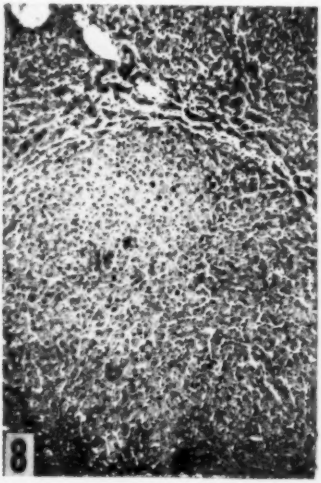
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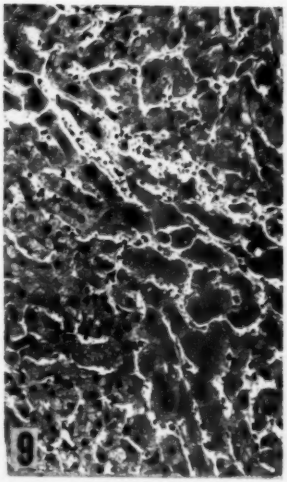
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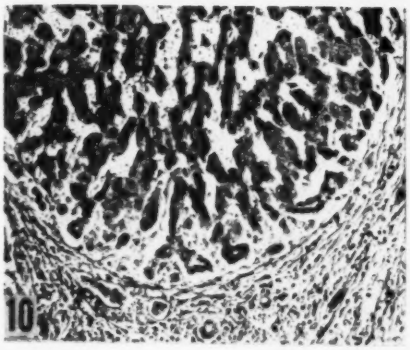
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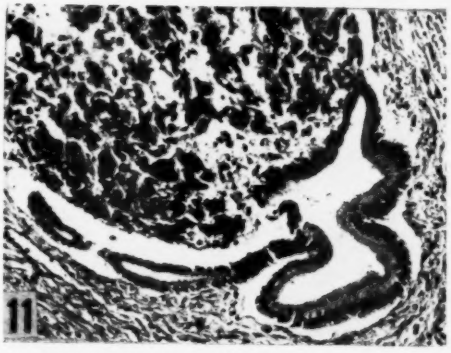
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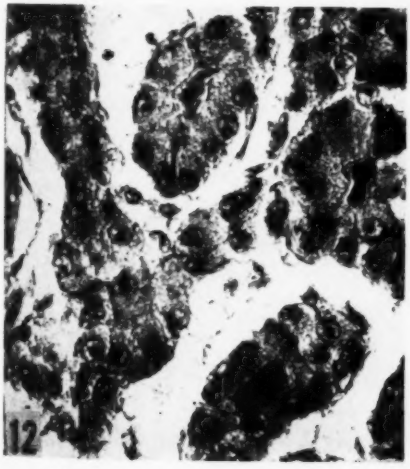
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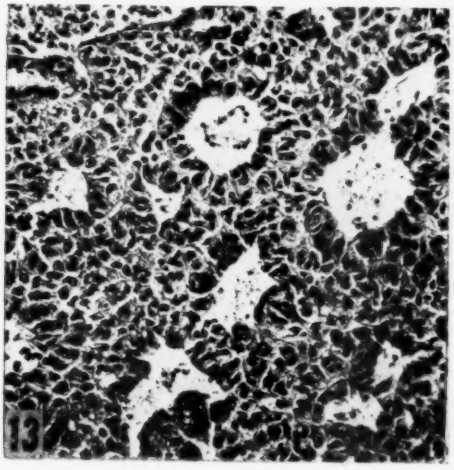
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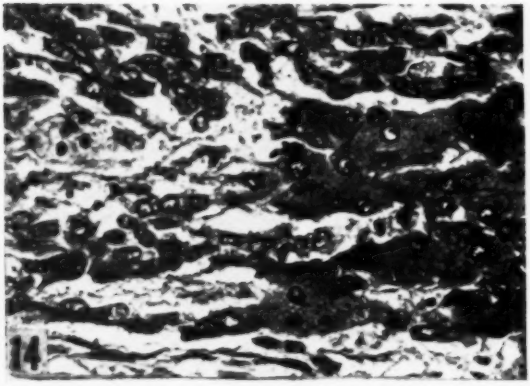
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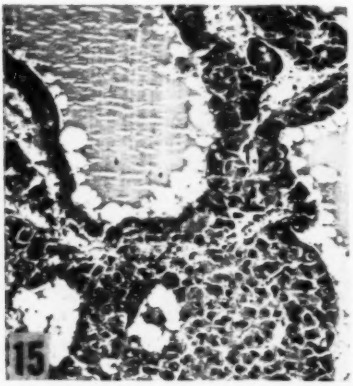
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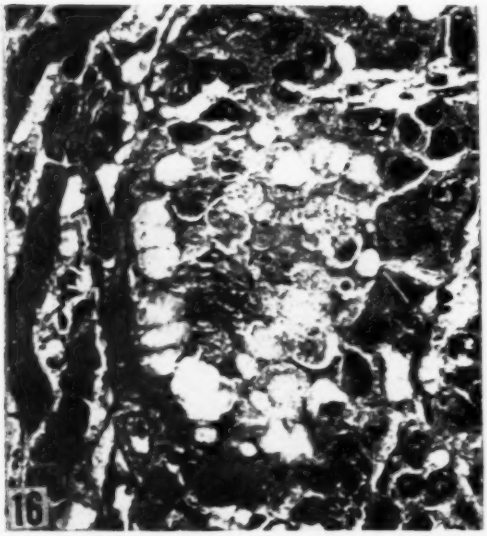
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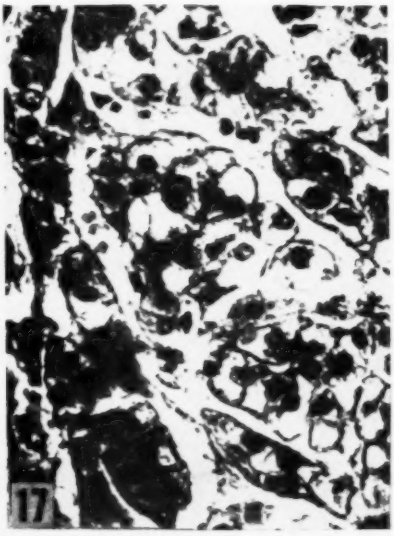
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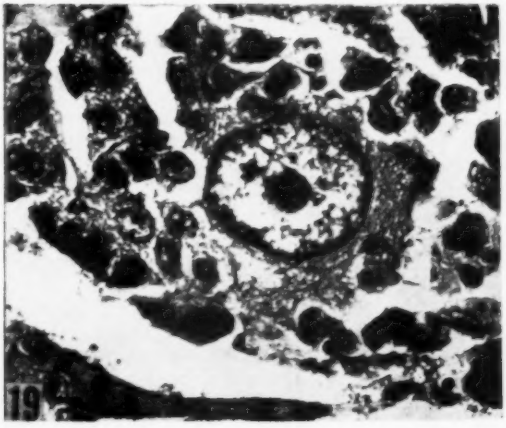
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General Histophysiology and Histopathology of the Liver[1]

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I shall first deal with a few of the relationships of liver injury, repair and cirrhosis to carcinogenesis and conclude with the discussion of some of the similarities and differences between the hyperplastic parenchymal nodule of compensatory regeneration and the neoplastic reaction of the hepatoma. Before discussing the relationship of liver injury to carcinogenesis, one must consider some of the features that are common to many types of liver injury as observed in experimental animals.

Liver Injury, Sites and Characteristics

In the experimental animal as well as in man, liver injuries may be induced by a wide variety of procedures. They may be placed in four main groups: toxic, infective, circulatory and deficiency-induced types. In many textbooks of pathology and reviews on the subject one may encounter lists of the various agents that come under the headings I have just cited with the author's or reviewer's opinion of the position within the liver lobule in which the injury is most frequently encountered. But such lists are probably of dubious value because even with a toxin as intensively studied as carbon tetrachloride one will find disagreement between investigators as to the site within the liver lobule attacked by the toxin. When disagreements by competent investigators of this sort arise, there is usually some basic reason for them and in this instance I think we do not have to look very far for a possible source of confusion. We have felt for a number of years that the classical concept of the hexagonal hepatic lobule is misleading and only a figment of the pig's imagination, and have recently published results of experiments carried out to define more precisely the functional unit of the liver (*1*, *2*). Dr. Rappaport, with co-workers in Toronto, and I have collaborated in some of these investigations.

Limitations in time and space preclude any detailed recounting here of the historical development of current concepts of the microanatomy of the liver. It would seem, however, that the classical concept of an hexagonal liver lobule largely neglects some of the fundamental relationships of the hepatic parenchyma to the anatomy of the blood vessels that supply and drain the parenchyma. In organs other than the liver, such as the kidney, the structural unit of the organ as most anatomists consider it is built around the *terminal* blood supply. The interlobular arteries in the cortex of the kidney define the boundaries of each renal lobule and no one would think of trying to construct the structural unit of the kidney around a larger vessel, such as the arcuate artery. But in the case of the liver an attempt to construct a unit on this basis seems to have confused investigators for years. The periphery of the classic liver lobule is indicated by portal triads comprised of branches of the portal vein, of the hepatic artery and of radicles of the bile duct ensheathed in a fibrous stroma. In a

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 11, 1954.

sense these large structures correspond to the arcuate artery and vein in the kidney because they only conduct blood to the parenchyma; they do not actually distribute it. What then are the *distributors* (rather than conductors) of blood to the hepatic parenchyma and will these serve adequately to orient the structural and functional unit of the liver?

From branches of the portal vein and hepatic artery in the portal triads arise smaller vessels that penetrate for a distance into the substance of the hepatic lobule. These small terminal vessels (usually of venous nature but may be either venous or arterial) are the functional distributing vessels. They indicate the center of the functional unit of hepatic parenchyma and the tissue immediately surrounding such a vessel is in every sense, *periportal*. Sinusoids are drained, of course, by the radicles of the hepatic vein which alternate between the terminal venules in a rather orderly fashion throughout the liver substance. In experimental dietary cirrhosis, the structural unit of the liver is dissected by the disease process because the fibrotic tissue surrounds these structural units. They constitute somewhat acinar structures because they form polygonal masses around each terminal venule. The appearance and relations of the units have been well defined and illustrated by Rappaport *et al.* in the references cited.

In our experience, almost every pathologic process produced by experimental means in an animal attacks the nonportal parenchyma farthest from the small terminal vessels that supply the center of Rappaport's functional unit of the liver. Therefore, these small terminal venules constitute highly important landmarks for orientation of lesions within this organ. An appreciation of the value of these venules as landmarks aids in the study of disease within the liver and in interpreting hyperplastic and regenerative changes in later stages. In the relatively intact regions surrounding terminal portal venules and arterioles regenerative changes in cirrhosis develop to compensate for the destruction at the peripheries of each structural unit near the radicles of the hepatic vein. The relation of the resulting nodules to multicentric foci of neoplasia in damaged livers will be considered later.

Small terminal vessels are not easily located unless looked for especially. By way of contrast, the portal triads with their vein, artery and bile ducts stand out very clearly even under the lowest magnifications of the microscope. The destructive processes that affect the nonportal regions of the liver farthest from these terminal portal venules may, however, abut against the portal triads, for as we have already demonstrated some portions of the portal triads lie midway between the sites of origin of the terminal portal venules and, therefore, may be just as nonportal (in a physiologic sense) as regions more immediately adjacent to radicles of the hepatic vein. Therefore, if observers insist on using these large portal triads as a guide for the distribution of lesions in the liver, confusion will undoubtedly persist. Some portions of these portal triads are at the peripheries of the structural units and some are very near their centers. Consequently, attempts to orient a lesion by using the portal triad as the landmark will give quite inconsistent and confusing results. The lesion, however, if related to the terminal venules will then be oriented with regard to the parts of the parenchyma that are supplied firstly or secondly, etc., by new blood reaching the organ. In our experience the destructive processes nearly always attack that portion of the parenchyma farthest from the entry of fresh blood and regenerative processes nearly always begin in portions of the parenchyma nearest the entry of fresh blood, *i.e.*, regions nearest the small terminal vessels I have demonstrated. These areas are adjacent to, or at one side of, a portal triad but they do not encircle the latter, and you will find many references in the pathologic literature to the para-portal position of foci of hyperplasia. No satisfactory explanation can be given for such a "para position" unless one has due regard to the small terminal vessels which again are the landmarks of the liver.

The fact that nodules of compensatory hyperplasia, and as we shall see in many instances, foci of neoplasia, develop adjacent to terminal portal vessels may be related to their unique blood supply. Breedis and Young (*3*) have reported that the blood supply of neoplasms in the liver of man, as well as in experimental animals, is almost

entirely arterial, confirming earlier work by Wright (*4*). The latter investigator suggested that growth of the tissue choked off the venules so that only arteries supplied the sinusoids. This peculiar arterial supply of liver tumors, either primary or secondary, may possibly explain the resistance of newly formed liver tissue to toxic agents. Following exposure of the liver of animals to various toxins, the newly formed compensatory parenchyma appears relatively insensitive to further administration of the toxin or the offending agent. We have observed that the parenchymal tissue in compensatory nodules of hyperplasia in choline-deficient rats rarely becomes as fatty as the parenchyma that exists in the liver from the beginning of the experiment. Wilson has shown in a crucial experiment (*5*) that the apparent resistance to toxic, infective and deficiency states exhibited by newly formed liver tissue may be explained by its vascular supply. He transplanted neoplastic liver tissue to subcutaneous positions and then on injecting toxins such as carbon tetrachloride discovered that this new tissue was just as susceptible to the toxin as the original liver tissue. The fact that the regenerative nodules and the hepatomas *in situ* are not injured while the liver itself and the tumor transplants are, is explained on the basis of blood supply rather than a difference in the tissues themselves.

In recent years the contributions of Elias have done much to clarify many of the true features of the anatomy and histology of the liver, both in the normal and diseased states. Little need be said here about the organization of liver cells into liver plates instead of liver cords. I think Elias' liver plates are now surely accepted by everybody. His observations on the formation of the fibrous tissue and sheets are elevating our concepts of cirrhosis from two-dimensional to three-dimensional planes. More pertinent to the topic of this conference are his observations on the organization of the liver cells in foci of hyperplasia. Newly formed liver tissue following injury reverts to an early embryonal characteristic because the newly formed liver tissue is organized in two-cell-thick plates instead of single-cell-thick plates, not only in a variety of experimental animals, including the rat, but also in man. As a practical pathologist I have found plate-thickness a most useful objective criterion for the identification of foci of hyperplasia.

I would like to say a word about fat within hepatic nuclei. It has been known from chemical studies of isolated nuclei (*6–8*) that fat is present in the nucleus. We have encountered the presence of stainable fat within the nuclei of liver cells in regenerating areas of hyperplasia in experimental cirrhosis in rats fed a low choline diet. More recently, Stowell and his group (*9*) reported the presence of stainable fat in most liver-cell nuclei under conditions of regeneration, hepatomatous growth and rapid growth of the liver in mice at one week of age. The Kansas investigators felt that the increase of nuclear lipids might be related to growth potential of the liver cell. During the past year, Blumenstein and Henry Best in my laboratory in Professor C. H. Best's department observed deposition of stainable fat in nuclei of liver cells in mice fed a low choline diet for relatively brief periods. Animals maintained on this diet for longer periods developed neoplasia without cirrhosis. I shall speak of these experiments in more detail later.

I have already mentioned some of the work of Stowell and this discussion would not be complete without further reference to his excellent investigations with techniques of ultraviolet microscopy and photometric absorption of nucleic acids in liver cells. He has reported among other findings (*10*) that nucleoli of liver cells under conditions of regeneration and of dietary protein repletion showed a significantly higher absorption of ultraviolet light near the nucleic-acid absorption bands, indicating a higher concentration of nucleic acids under these circumstances. He has demonstrated an increase in the size of the nucleolus in protein depletion, in regenerating liver cells and and in liver cells exposed to a carcinogen before malignant transformation occurred. All these findings plus the lipid nucleolar changes suggest that fundamental alterations in nucleoli, both in precancerous conditions and cancerous conditions may be of importance in understanding the nature of liver-cell growth.

The Relationship of the Hepatoma to the Hyperplastic Nodule

Most students of human pathology, including Ewing, Boyd, and many others, divide types of liver tumors into hepatomas or liver-cell tumors, cholangiomas or bile-duct cell tumors and finally mixed tumors containing both types. The exact definitions of hepatoma and cholangioma are confused, some employing the term hepatoma to describe only a malignant tumor and others modifying the term, speaking of benign hepatoma and malignant hepatoma. Both the liver-cell tumors and bile-duct cell tumors are further subdivided on the basis of their gross appearance as well as their microscopic appearance and as to whether they form adenomatous arrangements or otherwise. Details of such classifications do not concern us here but some of the studies of the pathogenesis of the tumors are of interest. Among the many classical investigations of the Millers are those in which they have studied the progressive microscopic changes in livers of rats treated with various carcinogenic azo dyes (*11*). The pathogenesis of tumors induced by these azo dyes was clearly demonstrated from bile-duct cells. The latter proliferate and gradually assume the characteristics of liver cells so that in this one instance we can see how artificial the classification of liver tumors into bile-duct cell types or liver-cell types can be. The fact that bile-duct cells may transform into growth identical with liver-cell tumors is complemented by the observations of Popper and his group, who have recently shown that proliferating liver cells in compensatory hyperplasia may form pseudocholangioles that very closely resemble bile ducts.

The differential diagnosis of hyperplasia from neoplasia in livers of both experimental animals and man is one that has perplexed and confused pathologists for many years. The close resemblance of small neoplastic foci in a cirrhotic liver to foci of hyperplasia irresistibly suggests that the former may arise from the latter. Such an event may actually occur but it is certainly not the only way in which neoplasia may arise in the livers of animals.

The pathogenesis of tumors in livers of choline-deficient mice is rather unusual. These animals develop very fatty livers with cysts that are filled with lipid and are relatively enormous, much larger in both absolute and relative terms than those encountered in rats or man. Despite the fact that mice have been maintained on these low-choline diets for periods of 5 or 6 months and despite the fact that foci of neoplasia develop in the livers of these animals, cirrhosis did not develop; yet tumors arose in these livers providing confirmation of Opie's observation (*12*) that it is not necessary to produce cirrhosis for experimental hepatoma formation. He obtained the two diseases separately in mice treated with butter yellow. Aside from the fatty change, the first indication that hyperplastic processes were involved in livers of choline-deficient mice was indicated by the formation of abnormal bile ducts. In these regions, which were nearly always in the center of the liver lobes (as distinct from the liver lobules), bile-duct proliferation went on until low-power fields were filled with the new structures. At this stage the term "adenomatous hyperplasia" seemed an apt description of the lesion. Although there was nothing in these lesions to suggest malignancy or even neoplasia *per se*, yet it was observed that these newly formed cells had invaded the radicles of the hepatic veins so that the tumor cells were found between the adventitia and the intimal endothelial lining of the vessel. So tenuous is the septum formed by the endothelium that separates these abnormal cells from the lumina of the veins that examination of the lungs of the affected animals was indicated and in one instance, metastases to the lungs were encountered. I will not say anything further concerning the pathogenesis of this type of dietary deficiency induced tumor because Dr. Copeland from Alabama is here and will discuss this more extensively later.

I have demonstrated these lesions, however, in an effort to show that under some conditions, at any rate, compensatory hypertrophy and hyperplasia can merge into neoplasia of even a malignant order without any sharp distinguishing criterion to differentiate the two.

Electron microscopy may be the tool that will afford us some of the answers to the questions I have formulated. Dempsey (*13*), by feeding silver to mice prior to their

sacrifice, has been able to stain in a sense the cells that he wishes to study under the electron microscope. Similar *in vivo* staining techniques applied to hepatoma cells with suitable controls might prove instructive. Dr. Fawcett will later tell us more about hepato-electronmicrography and I will refrain from encroaching further on his subject.

There is no doubt from both experimental and clinical evidence that cirrhosis is a precancerous condition but we question the value of biochemical and enzymatic studies of liver tissue from animals that have been given carcinogens or subjected to procedures that induce liver cancer in aiding our understanding of carcinogenesis. The precise differentation between neoplastic and normal liver cells on a morphologic basis can be especially difficult even for an experienced pathologist as Stowell (*10*) has pointed out. He cites cases from mice fed *p*-dimethylaminoazobenzene in which small nodules grossly resembling tumors turned out to be areas of fibrosis containing non-neoplastic proliferating bile ducts. He has shown that morphologic volumetric analysis by Chalkey's ratio method of some undoubted tumors indicated that they contain as little as 10 percent hepatoma cells and as much as 60 percent connective and vascular tissues. It would seem that such biochemical and enzymatic studies must be done on a cellular level with microtechniques such as those now being employed by Lowry (*14*) or else that we must obtain pure cultures of true tumor cells. Ham and his associates have developed a technique of the latter type whereby they have grown pure cultures of Novikoff tumor cells in chick allantoic sacs. They have transplanted these tumors through 50 or 60 passages now, and histologic examination reveals that they are composed almost entirely of pure tumor tissue with very little stroma, vascular, or fibrous tissue of any type.

I feel that I have encroached on the subject matter of many of the speakers to follow. Dr. Fawcett is to speak on electron microscopy of the liver cell, Dr. Copeland on induction of liver tumors by dietary deficiency and Dr. Miller on biochemical investigations on hepatocarcinogenesis. I hope they will forgive me if I have trespassed on their premises. The topic assigned to me was so general that I could pick out only a few points which I hoped might be of general interest.

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Observations Concerning Fluid Compartments, Blood-Flow Patterns and Bile-Formation in the Isolated Rat Liver [1]

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It is the purpose of the present remarks to supplement Dr. Hartroft's discussion of the morphologic aspects of the hepatic circulation by calling attention to some recent observations concerning the relation between blood flow through the liver and certain functions of the liver. Most of these observations have been made in isolated rat-liver preparations at the Naval Radiological Defense Laboratory.

In the course of studies concerning the mechanics of bile-formation in the isolated rat liver (*1*) it was observed that bile flow varies but little with perfusion pressures or with blood flow through the liver once a critical pressure region (50 to 60 mm. of H_2O) is exceeded; at lower pressures bile flow falls rapidly toward zero. The region of marked dependence of bile flow on blood flow or blood pressure is also the region in which the blood-flow/blood-pressure diagram shows the greatest curvature; this is the region between the nearly flat initial portion, where blood flow increases extremely slowly with increasing perfusion pressure (from 0 to, say, 30 mm. of H_2O), and the region of virtually linear increase of blood flow with increasing perfusion pressure (from 70 mm. of H_2O up). We hypothesized that both of these phenomena, the bile-flow change and the curvature of the flow-pressure diagram, might reflect changes in the distribution of blood throughout the vascular bed of the liver in this particular pressure region.

The possibility that the flow-pressure curves observed were an expression of anomalous viscosity of the blood under the conditions of our experiments, could be ruled out when it was shown that cell-free perfusion media yielded flow-pressure diagrams similar in character to those found with whole blood, though the region of curvature was shifted to lower pressures. These experiments, incidentally, were carried out under oxygen partial pressures of two atmospheres, in the experimental pressure chamber at the Naval Medical Research Institute (Bethesda, Md.) to assure adequate oxygenation of the tissue even in the absence of erythrocytes (*2*). Three lines of experimentation are being pursued at present in order to further explore what information concerning the hepatic vascular bed and concerning bile-formation can be derived from studies of blood flow in the isolated liver.

In the first place, changes in weight of the perfused organ are being studied. Results to date show close similarity between the curves relating blood flow and bile flow, and blood flow and liver weight (perfusion pressure being the only independent experimental variable). Indeed, if correction is made for what we assume to represent elastic distention of hepatic vessels as blood pressures in them increase, the flow-weight curves can be superposed precisely on the flow-bile production curves. This

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[2] The opinions and assertions contained in this report are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. (Article 1252, U. S. Navy Regulations, 1948.)

observation would accord well with the above hypothesis: The "corrected weight" is then a measure of the volume of blood in a hypothetical hepatic vascular tree in which all vessels are distended to a constant degree. To a first approximation we feel that this value is proportional to the number of sinusoids perfused. The correlation of weight with bile flow under these conditions suggests an all-or-none character for bile production with respect to blood flow. Bile production in any region starts when a critical blood supply is provided, and is not affected by further increases in blood flow through that region.

Such a concept draws further support from observations with preparations injected with India ink during perfusion at low pressures. Under these conditions the pigment is found histologically only in regions corresponding to the proximal region of Rappaport's acini (*3*). At higher perfusion pressures, a more even distribution throughout the tissue is found. Thus once again, perfusion at low pressures results in a distribution of blood flow demonstrably different from that at higher pressures, in accord with the original hypothesis.

Mathematical analysis of this situation is now being attempted. While not yet complete, promising results have been obtained using as a model a circulation consisting of a population of parallel vessels of equal resistance and of limited elasticity, distinguished from one another solely by differences in opening pressures. In an untreated isolated liver these may follow a nearly normal frequency distribution, with a median opening pressure around 35 mm., and a fairly narrow range (66 percent) fall within ± 15 mm. of the median.

The last group of studies related to the above hypothesis involves the effect of epinephrine or of norepinephrine on the hepatic vascular bed. These compounds result in instant and marked reduction in blood flow through the liver at constant pressure. However, by comparison with the same organ in the absence of epinephrine-blood flow being reduced to the same degree by decreasing the perfusion pressure, the epinephrine-treated liver produces much less bile and has a much lower weight. If bile flow in such a preparation is plotted against weight, points for the epinephrine-induced situations fall nearly on the same curve as those for the epinephrine-free preparation controlled by perfusion pressure alone. Again, liver weight, and presumably blood volume in the liver, are closely related to bile flow; once more, the assumption of a selective distribution of blood in the liver would readily accord with experimental fact. Indeed, analysis of flow-pressure curves during continuous epinephrine infusions, of India ink preparations, and of chromic phosphate colloid extraction coefficients (related to linear velocity of blood in the sinusoids), all fall in line with the hypothesis that the population of sinusoids, under the influence of epinephrine, changes its opening pressure distribution (flatter distribution, higher median), and little else.

From our point of view, further studies in this area are likely to be concerned with extending the mathematical treatment, with careful analysis of alternative interpretations, and with experimental exploration of the meaning and the consequences of the all-or-none relation of bile flow and blood flow suggested by present results. I should like to leave with those of you who are essentially morphologists two questions arising from this work: in the first place, how about vasomotion as a factor in the distribution of blood in the hepatic vascular bed? The Rappaport-Hartroft acini, it seems to me, have no inherent need for such a mechanism; our observations would appear readily to agree with theirs. Finally, in a recent letter to me Drs. Maegraith and Andrews mention that in their experience stasis in any visible hepatic sinusoid is a sign of liver injury—not of normal vasomotor activity. In the second place, I should like to ask whether in thinking about functional units of the liver we might not wish, in the light of the suggested all-or-none relation, to go even below the acini, and go on to a unit consisting of a sinusoid with its adjacent parenchymal cells, bile capillaries, and collecting duct. These would then correspond to the mathematical units in terms of which we have been thinking. I wonder whether an acceptable morphologic description of such a unit is feasible?

I should like now to turn to the relation between the vascular and the perivascular or interstitial spaces in the liver. It has been known for some time that lymph draining from the liver bears a remarkably close resemblance to plasma flowing through this organ at the same time, not only in regard to the crystalloid components, but also in regard to the total protein concentration, A-G ratios (*4*) and even in regard to the relations of some of the individual plasma proteins (*5*). Indeed, we have found that this close resemblance of liver lymph to blood plasma persists even on electrophoretic analysis of these fluids (*6*). It has never been clear, however, what particular anatomical component is being sampled by the collection of liver lymph. In a recent series of experiments, Mr. R. J. Holloway of our laboratory has extended previous observations concerning the distribution spaces of plasma proteins and of cellular elements of the blood within the liver. His studies have shown a distribution space for Fe^{59}-labeled erythrocytes of only 5 percent of the liver parenchyma, while that for iodinated albumin, as well as that for Cr^{51}-labeled whole rat plasma, was found to average close to 15 percent. Other investigators, faced with somewhat similar figures (*7*), have interpreted them to indicate a gross disparity between the hematocrit in the small vessels of the liver and that in the large vessels supplying the organ. In the present series of experiments, however, it was possible to obtain continuous recordings of the level of radioactivity in the organ following the administration of the radioactive materials. These observations showed a rather marked discrepancy between the very rapid, almost instantaneous, rise of radioactivity in the organ following the administration of Fe^{59}-labeled erythrocytes, and the steady radioactivity plateau maintained thereafter, on the one hand, and the much more gradual accumulation of activity and the slight positive slope of the ensuing plateau in organs perfused with plasma containing iodinated human serum albumin or with chromium-labeled plasma proteins. These observations suggest that the spaces measured by the two labels are by no means the same, and rather urge an alternative interpretation of the divergent quantities observed: According to this interpretation the true intravascular space is that which delimits the distribution of the cellular elements, while the plasma proteins, not retained by the endothelial walls, rapidly diffuse throughout the entire extracellular space within the liver. Additional credence is given to such an interpretation by comparison of the "colloid space" measured in this fashion with the sodium space measured by Manery and co-workers (*8*) and confirmed by us. This latter space ranges from 17 to 20 percent of the entire hepatic parenchyma, averaging approximately 18.5 percent. It would appear, therefore, as though the distribution space for iodinated albumin amounted to almost 90 percent of the total extracellular space in the liver. Indeed, if from these figures a mean concentration of plasma proteins in the extravascular fluid be computed, the figure thus arrived at is substantially identical with that found in previous experiments (albeit in the dog) for the concentration of plasma proteins in liver lymph in relation to those in blood plasma. The rapidity with which the iodinated albumin distributes itself throughout the entire space available to it, although smaller than that for the distribution of erythrocytes throughout the vascular tree in the liver, is high enough to suggest that it is no longer permissible to interpret the hepatic circulation in terms of the flow of blood through a closed vascular tree; rather it would appear as though a new concept would have to be evolved to accommodate what appears to be, in fact, an extravascular circulation of fluid, electrolytes, and plasma proteins in the liver. Using complex perfusion methods, we are now exploring the kinetic aspects of these relations to provide a baseline of quantitative information concerning the relative rates at which the different components come to equilibrium within the various compartments. Apart from this dynamic aspect of the subject, I should like to call your attention to the problem posed by the very existence of a large colloid distribution space so strongly suggested by these experiments; perhaps you would like to comment concerning the anatomical counterpart of this space. Since the slaying of the Disse space by Dr. Popper, I have found it exceedingly difficult to accommodate all this fluid in the tissues; I submit that the structures surrounded by Glisson's sheath provide at best an unlikely reservoir for this fluid in view

of the absence of any evidence of extraordinary permeability for the vascular endothelia in that region.

Finally, I should like very briefly to discuss the chemical and physical relations between the major fluid compartments of the liver. Studies in the isolated liver involving the use of crossover circulations, and of various isotopically labeled compounds have strongly suggested that bile flowing from the liver is closely related in composition and in origin of its components to the plasma flowing past the hepatic parenchyma, rather than to the composition of the hepatic parenchymal cells themselves (*9*, *10*). These observations indicate a very brief lag indeed between plasma and bile; I do not believe that they can be accounted for in terms of a lag in a branching duct system such as can account for some of the observations that have been advanced in regard to the renal tubular system (*11*). We are therefore confronted with a choice: We might postulate a chemically or a physically discrete, small active compartment in individual hepatic parenchymal cells, and assume that it is this compartment which is related closely to blood and bile composition and which, in turn, stands in somewhat more indirect relation to the rest of the contents of the same cells. Alternatively, we might postulate a mechanism by which a wide range of components can be exchanged rapidly between blood and bile formed by the parenchymal cells. While in the case of ions like sodium and potassium such an assumption might seem chemically feasible (concentrations in blood and bile are very close to each other under all experimental conditions), in the case of pigments and dyes like sulfobromophthalein this latter assumption becomes grossly untenable, since the thermodynamic activity of BSP or its derivatives in the biliary tree is between 1,000 and 10,000 times as high as that presumably in "equilibrium with it" in the blood plasma. On the anatomical side, two structural situations might permit such a secondary equilibration of blood and bile: in the first place, the peribiliary plexus described by various authors (*12*), and, in the second place, the recently described juxtasinusoidal extensions of the bile capillaries (*13*). With regard to the former, attention might be called to the fact that according to most observers the peribiliary plexus is supplied by the hepatic arterial system rather than by the portal venous system, while the data upon which the above considerations have been based were derived to a considerable extent from isolated organ preparations in which a hepatic arterial blood supply is not employed. With regard to the latter concept, the controversy of some years ago between Grafflin and Hirt (*14*) comes to mind in which apparent intracellular extensions of the biliary system, also outlined by fluorescein, were described by the latter and ascribed to radiation artefacts by the former. I believe that the errors inherent in Hirt's work have been minimized by the Swedish workers, but I am not aware of any specific studies undertaken by that group with the objective of eliminating all possibility of such artefacts. In all, I believe that the evidence now available does suggest the existence of the equivalent of an active compartment in the parenchymal cells, and the question which I would like to leave with this meeting is whether we can find a cytological counterpart of an entity which, from a physiological and functional point of view, seems quite real and convincing.

I have tried to present to you three sets of physiological observations which, for their interpretations, call for careful evaluation and, possibly, extension of existing anatomical and cytological concepts. It is my hope that collaboration between the physiologists and the morphologists in the future may serve to elucidate these, and to unfold and clarify a host of other problems which ultimately may contribute toward an understanding of the problems of the functioning of the liver tissue as a whole.

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Addendum

Since the original presentation of this communication, experimental and theoretical analysis of the circulation of the isolated rat liver has been substantially completed. The same opening-pressure distribution curve has been found to account satisfactorily for changes in hepatic vascular resistance, in dynamic hepatic blood volume, in liver weight, in bile-flow rate, and in colloid extraction efficiency, as functions of perfusion pressure or of perfusate flow [*cf.* (2) and (15)]. The concept of the hepatic vascular bed outlined in the present communication, therefore, appears to provide an adequate basis for the description of a wide variety of circulatory phenomena in the normal liver.

Observations on the Cytology and Electron Microscopy of Hepatic Cells [1, 2]

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In their efforts to correlate changes in the structure of the liver cell with alterations in its functional activity, cytologists have traditionally depended upon such criteria as the size of the nucleolus, the form and number of the mitochondria, the character and location of the Golgi complex and the quantity and state of aggregation of the basophilic substance of the cytoplasm. The electron microscope has so greatly extended the range of visible cell structure that possible functional variations in the submicroscopic organization of these several cell components are now beginning to command attention. The major features of the fine structure of the liver have been well described by Dalton *et al.* (*1*) and by Bernhard and his associates (*2*, *3*). Because of recent technical advances in fixation (*4*) and microtomy (*5*), the present study has been able to supplement these earlier observations with a more detailed description of the nature of the bile capillaries, the mode of attachment of hepatic cells to each other and their relation to the blood stream. Further observations have also been made upon the structure of the mitochondria, Golgi complex and the ergastoplasm or basophilic substance of the cytoplasm, and on the changes which these organelles undergo during fasting and refeeding.

Materials and Methods

The observations which follow are based upon examination of livers from 2 frogs (*Rana pipiens*), obtained commercially, and 10 rats of the Sprague-Dawley strain. Two of the rats were controls in a normal nutritional state, 2 were killed after 5 days of fasting, and 4 other animals were fasted 5 days and killed after refeeding on a high protein diet (fresh beef liver) for 4, 8, 16, and 24 hours, respectively. Two rats were fasted 5 days and refed a high carbohydrate diet (bread) for 8 and 26 hours, respectively. Small pieces of liver (1 × 1 × 2 mm.) were fixed by immersion for 4 hours in 1 percent OsO_4 buffered with veronal acetate to *p*H 7.5, according to the procedure of Palade (*4*). The tissue was washed briefly in distilled water, dehydrated in increasing concentrations of ethyl alcohol and embedded in *n*-butyl methacrylate (*6*). The blocks were sectioned with a thermal expansion microtome (*5*). Sections of approximately 0.03 μ to 0.05 μ were selected by using their interference colors to judge their thickness. The sections were examined, without removing the plastic, by the use of

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[3] A major portion of this work was done while the author was a Visiting Investigator of the Rockefeller Institute for Medical Research in the Laboratory of Dr. Keith R. Porter.

[4] Studies carried out in part during the author's tenure of a Lederle Medical Faculty Award.

the Phillips EM-100 or RCA model EMU-2B with a 30 μ objective aperture. Electron micrographs were made at original magnifications of 2,000 to 9,000 and enlarged photographically to the desired size.

To facilitate interpretation of the micrographs, blocks of the same livers were fixed for light microscopy in Zenker's or in Bouin's fluid, and sections were stained with eosin and methylene blue, hematoxylin and eosin, or chrom-alum hematoxylin and phloxine (7).

For the reader whose personal experience has been confined to light microscopy it may be helpful to comment briefly upon some consequences of the methods of specimen preparation for electron microscopy and to direct attention not only to the advantages but also to certain limitations of the study of ultra-thin sections at high magnification.

With the light microscope, observations can be made on the distribution of nuclear chromatin, the size of the nucleolus, the general character of the cytoplasm and the amount of chromidial substance, but little additional detail can be made out in sections prepared by routine methods. To reveal the mitochondria, Golgi complex, glycogen or lipid inclusions, separate fixatives and selective staining procedures are required. On the other hand, all of these constituents of the liver cell are preserved by 1 percent osmium tetroxide, the single fixative now in routine use for electron microscopy. There is no staining except that which results from the differential binding of reduced osmium by the components of the cell. The identification of the various organelles and inclusions depends largely upon recognition of their characteristic density and their submicroscopic structural organization. There are several disadvantages inherent in the present method of specimen preparation and examination which somewhat complicate the interpretation of electron micrographs. First, there is usually some compression of the section as the glass knife passes through the plastic block and this results in a slight distortion of shape. Hence, spherical structures, such as hepatic-cell nuclei, are apt to have oval or elliptical contours in electron micrographs. Secondly, the extreme thinness of the sections used (300–500 Å) creates problems in the interpretation of form which are not encountered in the examination of sections 100 times thicker (3 μ–5 μ) with the light microscope. Thus, the shape of a mitochondrion (0.5 μ × 3 μ) can be judged, whatever its orientation in a histologic section (5 μ), by changing the plane of focus of the light microscope. In an ultra-thin section (0.05 μ) viewed with the electron microscope one sees only thin slices of mitochondria, and a mixture of round, oval and elongated contours is observed (fig. 8) which does not indicate a variation in the shape of the mitochondria but is merely a reflection of their differing orientation with respect to the plane of the section. Thirdly, because the sections are examined through extremely small openings in a copper grid (120 mesh), the area in view at any one time is so restricted that the location of the field within the liver lobule cannot always be ascertained. Thus, regional variations in cell structure are difficult to establish and, as between livers of normal and experimental animals, one can seldom be confident that cells from corresponding parts of the lobule are being compared. Finally, the acid fixatives used for light microscopy penetrate quickly to the center of sizable blocks, but osmium tetroxide penetrates poorly and even in very small blocks there is a marked gradient in the quality of the fixation from the outside toward the interior. Although the *p*H and duration of fixation can be made uniform, there is at present no good way to be sure that the sections from the control and the experimental livers come from comparable depths in their respective blocks. Therefore, differences in the character of the fixation can easily be mistaken for changes related to the experimental treatment.

Observations

The Appearance of the Liver Cell with the Light Microscope

The cells of rat liver are typically polygonal in shape. The nucleus is round, has a smooth sharp outline, and contains one or more prominent nucleoli and a few small

clumps of chromatin that are often situated close to the nuclear membrane. The cytoplasm presents a mottled appearance, with coarse angular masses of basophilic material alternating with pale or unstained areas (fig. 1). The basophilic bodies are known to be rich in ribonucleic acid (*8*) and the paler areas of the cytoplasm can be shown to contain glycogen. The mitochondria are very numerous and after appropriate cytologic staining procedures they appear as plump rods or slender filaments, depending upon the physiologic state of the animal and location of the cell within the hepatic lobule (fig. 3). The Golgi complex is commonly found at the periphery of the cell near the bile canaliculus, but it may be juxtanuclear in position (*9*). The bile canaliculi appear as minute intercellular openings situated midway along the contact surfaces of adjacent cells. The cells are arranged in plates or laminae (*10*) one cell thick, and their principal support seems to be a loose feltwork of reticular fibers that is interposed between the parenchymal cells and the cells lining the sinusoids (fig. 4).

With minor modifications the same description can be applied to the frog liver (fig. 5). In this species the cells are arranged in laminae at least two cells thick (*10*) and the bile canaliculi tend to occur at the corners where four cells meet. Although the cells are quite large, their filamentous mitochondria are definitely smaller than those of the rat and somewhat fewer in number. The Golgi complex forms a very conspicuous network situated at the secretory pole of the cell in close association with a bile canaliculus (figs. 9 and 10).

The Finer Structure of Hepatic Cells as Revealed by the Electron Microscope

Nucleus.—In electron micrographs of rat liver cells the nucleus is often slighly deformed to oval shape. The karyoplasm is preserved, for the most part, in the form of very fine granules of uniform size (150–200 Å) and moderate density. These granules are thinly dispersed in some areas, while in others they are aggregated into ill-defined gray masses of irregular outline. The distribution of these darker areas within the nucleus corresponds fairly well with the pattern of chromatin seen in stained sections with the light microscope (fig. 8), and it is possible that the component particles consist of deoxyribonucleoprotein. A second kind of granule, less abundant and more osmiophilic than those just described, is found in small clusters of varying size (80–240 mμ) scattered through the karyoplasm. The nucleolus is comprised of a coarse strand (125–200 mμ in thickness) that branches and anastomoses to form a tight meshwork or tangled skein. This structure is in agreement with Estable's (*11*) description of the appearance of the nucleolus in several different cell types studied with the phase-contrast microscope. The coarse strand, called by Estable the nucleolonema, forms an unusually compact network in the nucleolus of the liver cell. When examined at high magnification with the electron microscope, it is found to be made up of closely packed, dense granules 150 to 200 Å in diameter. These granules bear a strong resemblance to the particles that are associated with the basophilic component of the cytoplasm and are presumed to be ribonucleoprotein (*12*).

The nuclear membrane or karyotheca consists of two membranes of nearly equal thickness (*circa* 90 Å) pursuing a parallel course 100 to 120 Å apart. The innermost membrane may appear to be thicker, owing to the accumulation of a layer of granular material on its inner surface. It is not entirely clear whether both membranes should be considered part of the karyotheca or whether only the inner layer is to be identified with the nucleus and the outer one regarded as the limiting membrane of the cytosome. Occasionally, the two membranes appear to be continuous with one another at the margins of small openings or pores (300 Å) through which there seems to be a free communication between the cytoplasm and the karyoplasm. A small area of rarefaction of the granular matrix of the nucleus is often found opposite one of these openings, suggesting either a local loss of electron scattering material from the nucleus or an influx of a substance of low density from the cytoplasm. These discontinuities in the nuclear envelope are very few in the liver compared with their occurrence in certain other cell types (*13–15*). Their significance is unknown, but it is tempting

to regard them as pathways for interchange of material between the nucleus and the cytoplasm.

Cytoplasmic matrix and its inclusions.—The cytoplasm presents a heterogeneous appearance with dense mitochondria and irregularly shaped basophilic bodies sharply outlined against a less dense background which has a fine granular character. Glycogen is quite uniformly dispersed throughout the cell instead of being precipitated in coarse clumps as it is after the alcoholic fixatives commonly used for its preservation in light microscopy. It has relatively little affinity for osmium and thus in micrographs of low magnification regions of cytoplasm rich in glycogen usually have a light gray, diffusely mottled character (figs. 7, 8, and 23). At higher magnification these areas may present a cloudy, amorphous appearance with no apparent fine structure or they may have a uniform ground-glass texture made up of many discrete 100 to 200 Å particles of low density (fig. 26). In other instances these particles are clumped to form slightly larger units which give the glycogen-rich areas of cytoplasm a coarser granularity. It is not known whether this variation in appearance is attributable to differences in the quality of the fixation or differences in the degree of polymerization of the glycogen. The small aggregates just described may correspond to the so-called "particulate glycogen" fraction isolated by differential centrifugation (*16*).

Small vacuoles are frequently observed near the bile canaliculi. Some of these are no doubt related to the secretion of bile. Others which contain dense granular material are probably deposits of lipochrome pigment. Droplets of neutral fat are rarely encountered in the normal rat liver but when they do occur they are small, spherical, and intensely blackened by the osmium. Lipid droplets are common in the hepatic cells of the winter frog, where they tend to assume bizarre myelin forms under the influence of osmic acid.

Basophilic substance or ergastoplasm.—The basophilic substance of the cytoplasm has been studied in electron micrographs by several investigators and its three-dimensional form has been variously interpreted. In earlier papers on the electron microscopy of liver, the basophilic substance was described as having a "filamentous" (*2*, *3*) or a "lamellar" (*1*) structure. In more recent work using improved methods of specimen preparation, the cytoplasmic basophilic component of cells has been found to consist of a complex system of intercommunicating, membrane-bounded tubules and vesicles (*12*, *17*). The term *endoplasmic reticulum* has been applied to this cytoplasmic organelle, and it has been found in one form or another in nearly all the cell types that have been examined to date with the electron microscope. In the liver cell the endoplasmic reticulum occurs in the form of tubules which pursue a meandering course through the cytoplasm, but in many places the tubules are expanded into broad flat vesicles. The former are circular or elliptical in section, while the latter have very long profiles and appear as pairs of parallel membranes about 200 Å apart that can be followed for some distance, but at the ends they are found to be continuous with one another. These laterally expanded and greatly flattened vesicles are referred to by Porter (*12*) as the "cysternae" of the endoplasmic reticulum and are called simply "ergastoplasmic sacs" by Weiss (*18*). The conspicuous basophilic bodies seen in liver cells with the light microscope are made up of large numbers of these in parallel array (figs. 14, 21, and 22), and they are essentially the same in the frog (fig. 7) as they are in the rat. The parallel arrangement of the flat vesicles within a basophilic body causes it to have a layered appearance in section, but it is important to note that the units of which it is composed are not solid sheets, as would be implied by the term "lamellae," but are hollow structures with a limiting membrane and a homogeneous content which differs in density from the surrounding cytoplasm. The outer surfaces of the membranes are dotted with large numbers of small dense particles (*19*) 150 to 200 Å in diameter (fig. 14), and these serve to distinguish the tubular and vesicular elements of the endoplasmic reticulum from other membrane-bounded structures in the cytoplasm. Fragments of the reticulum, together with its associated small granular component, are thought to constitute the "microsome fraction" of the biochemist, and there is good reason to believe that the small dense granules alone make up the

macromolecular particulate fraction recently isolated with the ultracentrifuge and found to be unusually rich in ribonucleic acid (*20, 21*).

Mitochondria.—The mitochondria are plump rods or short filaments randomly oriented in the cytoplasm. As previously noted by Bernhard *et al.* (*3*), they tend to be closely associated with the basophilic component of the cytoplasm. Individual mitochondria are very commonly partially encircled by a strand of the endoplasmic reticulum. A number of mitochondria are often closely applied to the outside of a basophilic body, and a few may actually be interleaved between its successive layers (figs. 21 and 22). The internal organization of rat liver mitochondria has been described in some detail by Palade (*22*) and the present study fully confirms his observations. Each mitochondrion is enclosed by a pair of membranes, of which the outermost is smooth and continuous, while the inner one is thrown up into narrow folds which project inward like a series of baffle plates (figs. 14, 15, and 16). These "cristae mitochondriales," as they have been called (*22*), are thought to be a means of increasing the area of the surface exposed to the amorphous matrix which occupies the interior of the organelle. As originally described, most, if not all, of these projections were believed to be broad-based folds or ridges, but in liver mitochondria many of them are actually slender cylindrical structures having a circular cross section. They appear to be less numerous than they are in mitochondria of many other metabolically active tissues. The mitochondria of frog liver are particularly simple in their internal structure, having only a very few slender villi projecting into the rather dense matrix in their interior (fig. 6).

Golgi complex.—At those sites in the liver cell where the Golgi material can be demonstrated by appropriate cytologic staining methods, the electron microscope reveals elaborate aggregations of parallel double membranes, small vesicles, and larger vacuoles containing from one to several coarse osmiophilic granules (figs. 11, 12 and 13). These peculiar membrane-limited systems resemble none of the forms commonly ascribed to the "Golgi apparatus" of classical cytology, but there can be little doubt of their identity. The same structural components, in similar relation to one another, have been demonstrated by Dalton and Felix (*23*) in the Golgi region of the cells of the epididymis and of a number of other epithelial cell types. The multiple layers of curving membranes in these structures do bear a superficial resemblance to the parallel double membranes of the basophilic bodies, but they are closer together and can also be distinguished by the fact that they lack the small dense particles which are usually an identifying feature of the basophilic component of the cytoplasm. Although quite variable in its organization, the very heterogeneity of the Golgi complex gives it a highly characteristic appearance, which is easily recognized in electron micrographs.

Changes in Fine Structure Associated with Fasting and Refeeding

After 5 days of fasting the liver cells are much smaller than normal, but there are no conspicuous nuclear changes and the mitochondria continue to be numerous. The cells still contain a considerable amount of glycogen and the number of lipid droplets in the cytoplasm is increased. The most conspicuous changes involve the basophilic component of the cytoplasm. It has been well known since the classical studies of Berg (*24, 25*) that the amount of basophilic material in the cytoplasm diminishes greatly during fasting. In recent years, the functional variations in the cytoplasmic basophilia of rat liver have been thoroughly restudied by Lagerstedt (*26*) using the light microscope, and rather detailed observations on the effects of fasting and refeeding have been reported by Bernhard *et al.* (*3*) using the electron microscope. The results of the present study are in full agreement with the findings of these prior investigations. After prolonged fasting, the basophilic bodies are no longer seen in histologic sections, and the parallel systems of membrane-bounded vesicles, which are their counterpart in electron micrographs, are found to have disaggregated and to have given way to individual strands of endoplasmic reticulum scattered throughout the cytoplasm. There appears to be a significant decrease in the total amount of endoplasmic reticulum present in the cell, as well as a change in its state of dispersion.

The double membranes which persist in the cytoplasm continue to have the usual complement of small granules on their surface and in other respects appear normal. Contrary to the impression which might be gained from light microscopy, the basophilic component of the cytoplasm apparently cannot be completely eliminated by acute starvation.

Within a few hours after the onset of refeeding on a high protein diet, ill-defined masses of material appear at the periphery of the cell (fig. 23). These are not observed in normal or in fasted animals and are believed to represent an early stage in the regeneration of the endoplasmic reticulum or basophilic substance. At higher magnification they are found to consist of a compact network of small vesicles and short tubules. These differ from the elements of typical endoplasmic reticulum in their closer organization and in that they generally lack the small dense particles on their surface. At longer intervals after the onset of protein feeding, dense reticular aggregations of this sort become increasingly prominent (figs. 24 and 25). They are much less conspicuous in the livers of animals refeeding on a diet consisting principally of carbohydrate. At the periphery of these irregular masses of vesiculated tubules their membranes are often seen to be continuous with those of neighboring strands of typical endoplasmic reticulum—a relationship which suggests that the tubules and flattened vesicles of the endoplasmic reticulum may arise by coalescence and reorganization of the small membrane-limited elements of these dense reticular formations. The small particulate component of the basophilic substance may then secondarily become associated with this membranous framework. During advanced stages of recovery from fasting, the small, dense particles occur abundantly in clusters lying free in the cytoplasm, whereas in both the normal and fasted livers such granules are largely confined to the surfaces of the reticulum. As recovery from fasting becomes complete, the flat sinuses or cysternae of the reticulum again become laterally associated in the parallel arrangement characteristic of the basophilic bodies of normal liver.

The changes in the form of liver mitochondria during inanition have been studied by Berg (*24*, *25*), Noel (*27*, *28*), McCurdy (*29*), and others with general agreement that the mitochondria cease to be filamentous and assume a granular or spherical form. In electron micrographs of fasting liver, all the mitochondrial profiles tend to have the same rounded shape. This is interpreted to mean that they have a nearly spherical form; for in normal liver where the mitochondria are randomly oriented rods and filaments, a section includes a wide variety of circular, oval, elliptical and elongated profiles. Soon after refeeding is started, the mitochondria resume their normal shape.

It is of interest that mitochondria that have rounded up in fasting have about the usual number of folds projecting into their interior and these are of approximately normal length. If enspherulation of the mitochondria were the result of swelling from imbibition of fluid, one might expect these structures to be shortened or effaced completely—just as folds in the intestinal mucosa are eliminated by distension of the organ. Although the mitochondria look larger when spherical, this seems to be due to the change in shape, rather than to any significant change in volume.

During both fasting and refeeding, mitochondria are occasionally found that appear to be divided into approximately equal halves by a transverse partition (figs. 18, 19, and 20). In micrographs of high resolution it can be seen that the septum crossing the middle is comprised of two closely approximated membranes each of which is continuous with the inner mitochondrial membrane (fig. 15). The interior of the organelle is thus divided into two separate compartments which are both enclosed within a common outer mitochondrial membrane. The resemblance of these paired structures to the blastomeres of a dividing ovum is very striking, and it is easy to jump to the conclusion that they represent a stage in mitochondrial division (fig. 17). They are not numerous, and careful search of very thin sections is required to locate them. In the present study of rat liver, about 20 clear-cut examples of this phenomenon have been observed. Two such mitochondria were noticed in frog liver and similar double mitochondria have been seen in rare instances in other tissues. The fact that they were first seen in livers of fasting animals and in those being refed, suggests that they may

be more numerous under those experimental conditions, but they certainly do occur in normal tissues as well.

Relation of the hepatic cells to each other.—In well-nourished animals the surfaces of adjacent liver cells appear with the light microscope to be smooth and in close apposition, except where they are grooved to accommodate the bile canaliculi (fig. 1). The factors responsible for the cohesion of their contiguous membranes are poorly understood, and investigators differ in their concepts of the mode of attachment of liver cells. An intercellular cementing substance has been postulated, but its presence is difficult to demonstrate. The loose argyrophilic reticulum (fig. 4), which is interposed between the parenchymal cells and the endothelial lining of the sinusoids, is generally credited with an important role in maintaining the normal cytoarchitecture of the liver. It has also been suggested that some degree of added mechanical stability results from the fact that the individual liver cells are enclosed in the meshes of a continuous network of bile canaliculi (*30*), but this involves the assumption that the bile canaliculi have a firm, resistant wall of their own, a point which has not been established beyond dispute. In teased preparations of fresh tissue, some workers have succeeded in isolating segments of bile canaliculi (*30, 31*), and, therefore, consider them to be tough, independent structures. Others still regard the bile canaliculus as an intercellular space and believe that its wall is merely a local modification of the opposing cell membranes (*32–34*).

The electron microscope reveals the cell surface clearly enough to settle some of these questions left unanswered by the light microscope. The hepatic cells are enclosed by a definite plasma membrane, and the membranes of neighboring cells appear to be separated by 100 Å to 150 Å. In this narrow interspace, no electron-scattering material is found which can be interpreted as intercellular cement. It can be argued that such a substance could exist and be of such a nature that it would not be preserved by osmic-acid fixation. On the other hand, where the distance between the cells is so small, it may be more logical to invoke long-range attractive forces between molecules to explain the intimate association of the membranes than to imagine an adhesive product of the cell which would be effective in so thin a layer. Coman (*35*) has made similar observations with the electron microscope on the relation of the hepatic cells and has likewise abandoned the concept of intercellular cement. He continues to believe, however, that calcium ions are somehow essential for maintaining cellular cohesion.

Normally the membranes of contiguous liver cells diverge from one another only at the site of the bile canaliculus. The latter has no separate wall or limiting membrane of its own but is, in fact, a tubular intercellular space formed by the apposition of corresponding grooves in the surfaces of the two adjoining cells. Thus, in rat liver, each of the two cells contributes one half of the wall of the canaliculus. In the frog where the bile capillary is often formed at the junction of three or four cells, each contributes a third or a fourth of its wall (fig. 11). That portion of the cell surface which forms part of the wall, is studded with short processes or microvilli which project into the lumen of the bile capillary. These are similar to the cell processes comprising the striated border of the intestine but are shorter. Where the plasma membranes come together at the sides of the canaliculus, they are slightly thickened and more osmiophilic than elsewhere (fig. 11). In the livers of rats fasted for 5 days the cells are less coherent than normal, and a slight separation between them can be detected with the light microscope (fig. 2). This tendency for loosening of the cells is probably accentuated somewhat by the osmic-acid fixation. Some degree of separation of the cells was observed in nearly all blocks of tissue from rats killed during fasting or in the early stages of refeeding. In electron micrographs of such material it is apparent that the liver cells are most firmly adherent near the bile canaliculus (figs. 27 and 28). Even when the cell surfaces are fairly widely separated elsewhere, they usually remain attached at the above-described thickenings of the cell membranes on either side of the bile canaliculus (fig. 31).

The slight separation of the cells in the experimental livers served to draw attention to another specialization of the contact surfaces of liver cells which appears to have

escaped detection with the light microscope. A short distance on either side of the bile capillary one or two stout, studlike processes can often be found projecting from one cell into concavities of appropriate shape in the surface of the neighboring cell (figs. 27 and 28). This cell in turn may have similar projections which exactly fit receptacles in the first cell. These processes are usually 0.5 to 1 μ long and 0.2 to 0.4 μ wide, but they vary somewhat in size. Not infrequently they are expanded at the tip and the concavities into which they fit are constricted around the base. An interlocking relationship results which is reminiscent of the mortise and tenon or dovetail joint of the cabinet maker. These structures are present in livers of normal as well as fasted rats, but due to the closer coaptation of the cell surfaces in the normal, they are somewhat more difficult to find. They have also been seen, but more rarely, in the frog.

Relation of the hepatic cells to the blood stream.—The exact relationship of the hepatic cells to the blood and lymph vascular systems is not well understood. The parenchymal cells of the liver are generally believed to be separated from the blood stream at all points by a continuous layer of cells lining the sinusoids. The exact nature of these cells is still unsettled. According to some authors, there are two distinct cell types, the phagocytic Kupffer cells and nonphagocytic endothelial cells. Others consider these to be different functional states of the same cell type. The controversial space of Disse, a narrow cleft that can sometimes be seen between the liver cells and the cells lining the sinusoids, is interpreted by some workers as a shrinkage artefact and by others as a real entity functionally important as a site of lymph formation and a pathway for its drainage. Several incidental observations have been made in the course of the present study which have a bearing upon these points of dispute in liver histology.

In electron micrographs the surfaces of the hepatic cells facing the sinusoids are often found to be covered with many short processes that are not seen with the light microscope (fig. 29). These are more variable in length and more disordered in their arrangement than the slender processes which comprise the striated border of the intestine, but in other respects they are similar. These surface modifications of the liver cell were more readily visualized in the experimental animals than in controls, and it is quite possible that they are rendered more conspicuous by fasting, just as the intestinal border is said to vary in its thickness in different nutritional states. Such villous processes are characteristic of free surfaces of epithelial cells. They are lacking at those points where the thick perinuclear portions of the lining cells are in intimate contact with the liver-cell surface; but the broad, thin, peripheral extensions of the lining cells make up the bulk of the wall of the sinusoid, and these appear to rest lightly upon the ends of the slender surface processes of the liver cells without being firmly attached. This relationship of the lining cells to the microvilli on the hepatic cells provides a basis for understanding the nature of the "space of Disse" and its variable appearance as seen with the light microscope. If the surface processes on the liver cells vary in length in different functional states, as we suspect, then in some conditions the lining cells would appear closely applied to the parenchymal cells with no "space of Disse" visible, while under other conditions, which favored longer processes on the liver cells, they would seem to stand off a short distance from the surface of the parenchymal cells.

In most places a thin layer of cytoplasm belonging to an endothelial or Kupffer cell can be made out between the liver cell and the lumen of the sinusoid (fig. 30), but it is not uncommon to find small areas in which the villous surface of the liver cells appears to be directly in contact with the blood stream without the interposition of endothelium (fig. 29). These apparent discontinuities in the wall of the sinusoid could be the result of shrinkage and retraction of the lining cells during fixation, but buffered osmium does not have this effect upon endothelium elsewhere and it is felt that the wall may actually be discontinuous or fenestrated. Some of the lining cells contain phagocytized material and others do not. The two categories, however, do not seem to differ sufficiently in their fine structure to be regarded as two distinct cell types.

In view of their loose attachment to each other and to the liver cells, one still cannot exclude the possibility that these lining cells are normally, or under certain conditions may become, a shifting population of more or less independent littoral cells instead of fixed elements of a continuous endothelial lining.

Discussion

The electron microscopy of the liver is subject to the same sources of artefact as light microscopy and its findings must be interpreted with the same caution, but the wealth of additional detail which it reveals has already clarified many issues left in doubt by the limited resolving power of the light microscope. Thus, the fine structure of the mitochondria and Golgi complex, the organization of the basophilic component of the cytoplasm, and the nature of the bile capillaries have been clearly established. The problem of the origin of cytoplasmic basophilia has been brought up again; the question as to whether mitochondria divide has been raised anew; and the long standing dispute over the continuity of the lining of the liver sinusoids has been reopened on the basis of new evidence.

Origin of cytoplasmic basophilia.—Both the nucleolus and the cytoplasm of the liver cell are known to contain ribonucleoprotein. In the restitution of cytoplasmic basophilia after fasting, many investigators have noted that the nucleolus enlarges before an increase in cytoplasmic ribonucleoprotein is detectable either by staining methods or ultraviolet absorption techniques. As recovery progresses the basophilic material tends to accumulate in the cytoplasm near the nucleus. From these observations it has been inferred by Caspersson (*36*), Lagerstedt (*26*), and others that the nucleolus contributes to the formation of the basophilic substance of the cytoplasm. Attractive as this hypothesis is, the evidence upon which it is based needs to be reappraised in the light of the new findings with the electron microscope, which disclose a number of ways in which the observations with the light microscope may be misleading. Neither staining nor absorption techniques are able to distinguish clearly between actual quantitative changes in ribonucleoprotein and changes in its state of dispersion. It is apparent from the study of electron micrographs that disappearance of cytoplasmic basophilic bodies from the liver cells in fasting is, in large measure, the result of disaggregation of the elements of the endoplasmic reticulum. Although there is also considerable loss of basophilic material, this is not so great as would appear to be the case when studied with the light microscope. Similarly, the enlargement of the nucleolus observed by optical methods involves a loosening of the meshes of the netlike organization of the nucleolonema and may not entail a proportionate increase in nucleolar substance. There is evidence that the basophilic material is displaced during refeeding both toward the nucleus and toward the cell membrane by accumulation of glycogen in the body of the cell. Its perinuclear distribution, therefore, does not necessarily mean that the basophilic material is being formed at the nuclear membrane. To the contrary, the electron micrographs of the present study indicate that at least the membranous framework of the basophilic substance tends to arise at the periphery of the cell. When it first appears it lacks the granular component in which most of the ribonucleic acid is thought to reside, and hence the incipient stages in reformation of the basophilic bodies are probably not detected by staining or absorption techniques. The small (150 Å) particles found both in the nucleolus and cytoplasm are thought to be macromolecular units of ribonucleoprotein, but it is still not known whether they arise independently in these two sites or whether those of the cytoplasm come from the nucleus and secondarily become associated with the membranes of the endoplasmic reticulum. The pore-like openings in the nuclear membrane described here provide a pathway through which particles of ribonucleoprotein could pass from the nucleus to the cytoplasm. Pollister *et al.* (*37*) believe that they have seen with the electron microscope compelling evidence for just such an interchange of material between the karyoplasm and cytoplasm through pores in the nuclear membrane of amphibian oöcytes.

The question of mitochondrial division.—In mitotic division, mitochondria are apportioned about equally between the daughter cells, and it is self-evident that cells of growing tissues would be progressively depleted of mitochondria if these did not at least double in number during interkinesis. Since this does not occur, they must multiply, but it is not known whether their number increases by division or whether they are formed *de novo* from submicroscopic elements of the cytoplasm. Little progress has been made with the light microscope toward a satisfactory solution of this problem. Transverse constriction and subsequent division of mitochondria in ciliates has been described in both living and fixed material by Fauré-Fremiet (*38*). Payne (*39*) has recently reviewed the morphological evidence for reduplication of mitochondria and presented photomicrographs of adrenal-cortical cells from fowls which clearly suggest mitochondrial division. The strongest evidence to date has come from observation of living cells in tissue culture where the behavior of cytoplasmic organelles can be observed directly and recorded cinematographically. Mitochondria studied in this way exhibit an extraordinary degree of plasticity which was not suspected from earlier work on fixed and stained preparations. They are found to be continuously in motion within the cytoplasm, displaying complex flexuous movements and changing their shape from moment to moment. They bud, branch, and fuse, and filamentous forms often divide transversely into two or more shorter segments (*40*, *41*).

As long as mitochondria were thought to be coacervates or simple myelin forms of lipoprotein (*42*), their changes of shape and their fragmentation or coalescence could easily be attributed to slight changes in the physiochemical state of the surrounding cytoplasm. Now that they are known to be heterogeneous structures, bounded by membranes and having a complex, internal organization, a more complicated mechanism must be invoked to account for their observed behavior. It is possible that the anomalous double mitochondria, described here for the first time, represent one stage in their reduplication by division. One can postulate that an annular fold, or crista, first arises from the inner membrane, and that the free edges of this advance until they meet in the middle, thus completing the partitioning of the interior of the mitochondrion and resulting in the double condition observed here. It is assumed that a local dissolution of the outer membrane would take place opposite the edges of the septum, permitting a separation of the two halves of the organelle between the leaves of the septum. A second membrane would then form over this surface and two complete mitochondria would result. To date, stages in the process other than the one illustrated in plate 7 have not been identified with certainty.

Many of the paired mitochondria described here were seen in livers of fasting animals. McCurdy (*29*) was of the opinion that the change in shape of mitochondria from filaments in well-fed animals, to granular forms in inanition, resulted from transverse division of the elongated forms. The septate mitochondria observed in the fasting state could be examples of this sort of division. On the other hand, mitochondria with a transverse partition were observed also during the period of refeeding when mitochondria were resuming their normal shape. Smith (*43*) was convinced that the formation of filamentous mitochondria results from a fusion of smaller spherical ones. The possibility that the double mitochondrial structures encountered in electron micrographs represent mitochondria in process of fusion certainly cannot be excluded. The correct interpretation of these structures must await further studies under experimental conditions which will permit one to distinguish more easily fusion from fission.

The nature of the bile canaliculus and the cohesion of liver cells.—The present observations on the bile canaliculus reaffirm the fact that it is not a separate entity with a wall of its own but is in effect an intercellular space. The delicate processes projecting into its lumen were described by Dalton (*1*) in electron micrographs of mouse liver, and there now seems little doubt that they were actually observed some time before that with the light microscope. Morton (*34*), in studying the large cells of axolotl liver, described an acidophilic modification of the hepatic cell surfaces lining the bile

capillaries and wrote that "throughout most of its thickness this consisted of cylindrical processes approximately 0.3 μ in diameter which were directed toward the center of the lumen," and elsewhere he referred to this as a "brush-like" border. The localized thickenings of the cell membranes on either side of the bile canaliculi apparently correspond to the *terminal bars* which were described in this location by Zimmerman (*44*) with the light microscope. Similar thickenings on adjacent cell membranes have been observed in intestinal epithelium just below the striated border where terminal bars are also seen with the light microscope (*10*). These osmiophilic plaques appear to be essentially the same as the thickenings of the apposing cell membranes found by Porter (*45*) at the points of end-to-end contact between the processes of neighboring epidermal cells. These were recognized by the light microscopists as the desmosomes or nodes of Bizzozero, in the middle of the so-called intercellular bridges. All of these seem to be examples of local specializations of the cell membranes to insure firmer cohesion between cells. Certainly the electron micrographs of the present study show clearly that liver cells adhere more tenaciously at the edges of the bile canaliculi than anywhere else on their surfaces of contact.

The significance of the studlike processes and corresponding indentations of the confronted cell surfaces described here for the first time is not immediately apparent. They are certainly not numerous enough to provide an important increase in the area of the surfaces. Their reciprocal arrangement and interlocking structure make it tempting to assign them an accessory role in maintaining the attachment of the cells in the region of the bile canaliculus. To have the mechanical stability for such a function both the process and its receptacle would need to have a firm consistency. This condition could be fulfilled if the cell possessed a firmly gelated cortex, but at present there is no evidence for the existence of a well-differentiated ectoplasmic layer on the liver cell. Indeed micromanipulative studies suggest that the consistency of liver cell cytoplasm is unusually soft (*46*). It is not known whether these processes are transient structures suddenly extended and as rapidly withdrawn or whether they have a certain degree of permanence. Nor is it known whether their number varies in different physiologic states or remains relatively constant. Until these points are clarified, one can only speculate as to their function.

Relation of the liver cells to the blood stream.—In recent years the old controversy concerning the nature of the wall of the liver sinusoids has largely subsided. The consensus seems to be that they have a continuous lining of which the Kupffer cells are an integral part. The widespread acceptance of this conclusion, however, does not mean that all the objections to it have been satisfactorily resolved. In continuous layers of endothelium elsewhere in the body it is relatively easy to impregnate the cell boundaries by the silver-nitrate method, but efforts to accomplish this in the liver have generally been unsuccessful even though endothelial cell outlines can be clearly demonstrated in branches of the portal and hepatic veins of the same preparation. This led to the suggestion that the wall of the liver sinusoid is syncytial. The same observation, however, could have led just as well to the conclusion that the cell outlines are not satisfactorily silvered because the cells do not meet edge-to-edge. Von Kupffer (*47*) and a number of later investigators reported that the phagocytic lining cells, when engorged with particulate matter, often come away from the wall of the sinusoid and migrate freely within the lobule (*48*), and that they can easily be dislodged from the walls of the sinusoids by perfusion (*49*). If these cells were part of a continuous endothelial lining layer, whether syncytial or not, it is somewhat unlikely that they would be so readily mobilized from their attachments. On the other hand, the loose association of the wall of the sinusoid with the surface of the liver cells which is revealed in electron micrographs is entirely consistent with the easy detachment of these cells.

The facility with which fairly large particles leave the blood and gain access to the interior of the liver cells also casts some doubt upon the concept of a continuous endothelial lining. The carbon particles of India ink do not normally escape through an intact capillary endothelium, but it has been shown that such particles injected into

the blood stream are taken up by the liver cells as well as the Kupffer cells (*48*, *50*).

Sharpey-Schafer (*51*) and Herring and Simpson (*52*) observed many erythrocytes within liver cells, and they concluded that the sinusoids are tissue spaces lacking a complete endothelial lining and that the liver cells in many places are bathed directly by blood. Not many workers have shared this extreme view, but the presence of erythrocytes within the cytoplasm of liver cells has been reported again and again in experimental animals after administration of a wide variety of substances, such as peptone (*53*), phosphorus (*54*), arsenic (*55*), urethan and allylformate (*56*, *57*). Intracellular erythrocytes have also been observed in the livers of dogs in anaphylactic shock (*58*) and in humans suffering from hemochromatosis (*59*), obstructive jaundice (*55*) and hemolytic jaundice (*60*). It cannot be denied that some degree of toxic damage to the endothelium may occur in all of these examples, but marked extravasation of blood elsewhere in the body is seldom reported in these conditions, and one is obliged to conclude either that the endothelial lining of the sinusoids is extraordinarily sensitive to such agents or that there are normally openings in the wall through which the liver cells are directly exposed to the blood. The evidence presented in the present paper is certainly not compelling enough to settle the question of the continuity of the lining of the sinusoids, but it is sufficient to reopen the question and to point out the need for further investigation of this old problem.

Summary

The hepatic cells of normal rats and frogs were studied with respect to the fine structure of the nucleus and nucleolus, and the mitochondria, Golgi complex, and basophilic bodies of the cytoplasm. The changes in the form of the mitochondria and cytoplasmic basophilic substance (endoplasmic reticulum) during fasting and refeeding are described. In the regeneration of the basophilic substance of the cytoplasm after fasting, its membranous framework appears first at the periphery of the cell rather than near the nucleus. The small granular component of the endoplasmic reticulum, which is thought to be rich in ribonucleic acid, becomes associated with the membrane surfaces later. It is considered possible that these small particles arise in the nucleus and reach the cytoplasm by passing through pore-like openings in the nuclear membrane. Mitochondria are described which are transected by a double membrane in a manner suggesting mitochondrial division. Studlike processes found on the contact surfaces of liver cells fit into concavities of corresponding shape in the adjacent cell and are believed to assist in maintaining attachment of the cells to one another. The surface of the liver cell facing the sinusoid is covered with delicate villiform processes. The endothelial cells appear to rest lightly upon the ends of these microvilli, and this loose relationship to the liver cell may account for the so-called "space of Disse" seen with the light microscope. The question of the nature of the lining cells and the continuity of the wall of the sinusoids is discussed.

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PLATE 104

FIGURE 1.—Photomicrograph of liver from a well-nourished rat. Large deeply stained clumps of basophilic substance are present in the cytoplasm. See figures 21 and 22 for the appearance of these basophilic bodies with the electron microscope. Eosin and methylene blue. × 900

FIGURE 2.—Liver from a rat fasted for 5 days. The cells are much smaller than those of the normal animal in figure 1 and contain no obvious basophilic bodies. Notice that the cells are only loosely attached and in places (see *arrows*) they are slightly separated. Eosin and methylene blue. × 900

FIGURE 3.—Rat liver showing normal local variation in the form of the mitochondria. In a few of the cells at the *upper right* they are spheres or short rods while in the rest of the cells they appear as slender filaments. Acid fuchsin-methyl green. × 900

FIGURE 4.—Liver stained to demonstrate the loose network of reticular fibers situated between the hepatic cells and the cells lining the sinusoids. Pap's silver stain. × 900

PLATE 105

FIGURE 5.—Photomicrograph of frog liver. Note the angular basophilic bodies and pale areas of cytoplasm occupied by glycogen. Areas similar to those enclosed in the squares A and B are illustrated in electron micrographs in figures 7 and 8, respectively. Chrom-alum hematoxylin and phloxine stain. × 900

FIGURE 6.—Electron micrograph of a mitochondrion (m) from frog liver. A double limiting membrane is visible and the interior is occupied by a homogeneous matrix of moderate density. There are only a few processes projecting inward from the membrane and these tend to be slender cylindrical villi. Near the mitochondrion are a number of strands of the ergastoplasm or endoplasmic reticulum (er). × 42,000

FIGURE 7.—A small area of cytoplasm from a frog liver cell including two basophilic bodies (bb) which consist of parallel arrays of flattened vesicles of the endoplasmic reticulum. Several mitochondria (m) are shown and the pale faintly granular background is glycogen (g). × 34,000

PLATE 106

FIGURE 8.—Electron micrograph of a portion of a frog liver cell (see square B in fig. 5 for orientation). The nucleus (n) is at the *upper left* and shows a nucleolus (nl) and a pattern of densities corresponding to the chromatin of light microscopy. The

double nature of the nuclear membrane can be seen at the arrow. The cytoplasm contains numerous mitochondria (m) and both parallel aggregations and isolated strands of endoplasmic reticulum (er). × 20,000

PLATE 107

FIGURES 9 and 10.—Photomicrographs from a section of frog liver prepared by the late Dr. Harold Weatherford by the Nassanov-Kolachev method × 900. The Golgi complex appears as a dense black network at the secretory poles of the cells, close to the bile canaliculi. An area such as that in square C is shown at higher magnification in figure 11.

FIGURE 11.—Electron micrograph of frog liver including a cross section of a bile capillary (bc) formed at the junction of three cells. Note the short villi extending into the lumen and the greater thickness and density of the cell membranes near the bile capillary (*). In two of the cells the Golgi complex (G) is shown in typical relation to the bile canaliculus. × 36,000

FIGURES 12 and 13.—Two examples of the structure of the Golgi complex as seen in electron micrographs of rat liver. It consists of paired parallel membranes, clusters of small vesicles and larger vacuoles containing coarse osmiophilic granules. × 40,000 and × 29,400, respectively.

PLATE 108

FIGURE 14.—Electron micrograph of several mitochondria (m) of rat liver. One is cut longitudinally and many "cristae" (cm) or folds of the inner membrane can be seen projecting into its interior. These are more numerous in the rat than they are in the mitochondria of frog liver cells. At this magnification, small dense particles (150 Å) are clearly visible on the membranes of the endoplasmic reticulum (er). × 55,000

PLATE 109

FIGURE 15.—A mitochondrion divided by a partition formed of two apposed layers of the inner mitochondrial membrane. At the points indicated by *arrows*, the outer membrane can be seen to continue uninterrupted around both halves. It is possible that this double condition is a stage in mitochondrial division. × 44,500

FIGURE 16.—Mitochondria which have assumed a roughly spherical form during fasting. This change in mitochondrial form does not noticeably affect the length of the cristae mitochondriales. Notice the double nature of the membranes at the arrows. × 48,750

PLATE 110

FIGURE 17.—An area of cytoplasm of a liver cell from a rat fasted for 5 days. Two of the mitochondria in this figure have a diplococcal form which could be interpreted as evidence of their reduplication. × 12,500

FIGURES 18 to 20.—Additional examples of mitochondria traversed by a double membrane in such a way as to suggest that they are dividing into two. The possibility that two mitochondria are coalescing cannot be excluded, however. These are from livers of animals refed after a period of starvation. × 42,250, × 32,300, × 32,500

PLATE 111

FIGURES 21 and 22.—Two typical cytoplasmic basophilic bodies (bb) from livers regenerating 4 days after partial hepatectomy. They are essentially the same as in normal, well-nourished rats. They have a layered appearance in section and consist

of paired membranes associated in roughly parallel array. Mitochondria (m) are closely applied to the surface of these aggregations and are sometimes included between the layers. × 12,125

FIGURE 23.—Liver cells from a rat fasted 5 days and refed a protein diet for 4 hours before being sacrificed. The cells are loosely attached except at the edges of the bile canaliculi. Some glycogen (g) and lipid (l) are present in the cytoplasm. The basophilic material is reduced to a few disaggregated strands (er). At the periphery of the cells are closely packed masses of small vesicles (*) which are believed to represent an early stage in the regeneration of the endoplasmic reticulum. × 7,800

PLATE 112

FIGURE 24.—Liver cells from a rat fasted 5 days and refed on a high protein diet for 8 hours. Observe the prominent irregular masses of material (*) from which strands of typical endoplasmic reticulum (*at arrows*) seem to be arising. × 6,100

FIGURE 25.—An area of liver-cell cytoplasm from an animal recovering from a period of fasting. The close reticular nature of the regenerating basophilic material can be seen. × 18,000

FIGURE 26.—An area similar to that delimited by the square in figure 25 shown at higher magnification. The ill-defined masses of material which appear in the cytoplasm during the early stages of recovery from starvation are comprised of a network of vesiculated strands of small membrane-bounded tubules (er). These generally lack the small granules which normally characterize the membranes of the fully formed endoplasmic reticulum. The pale granular material in the background is glycogen (g). × 55,000

PLATE 113

FIGURE 27.—Electron micrograph of part of the junction between two liver cells which are slightly separated except at the edges of the bile canaliculus. A studlike process of the *lower* cell projects into a concavity in the *upper* cell. This process is expanded at its tip, and the concavity is constricted around its base in a manner suggesting that this is a device for cell attachment. × 16,500

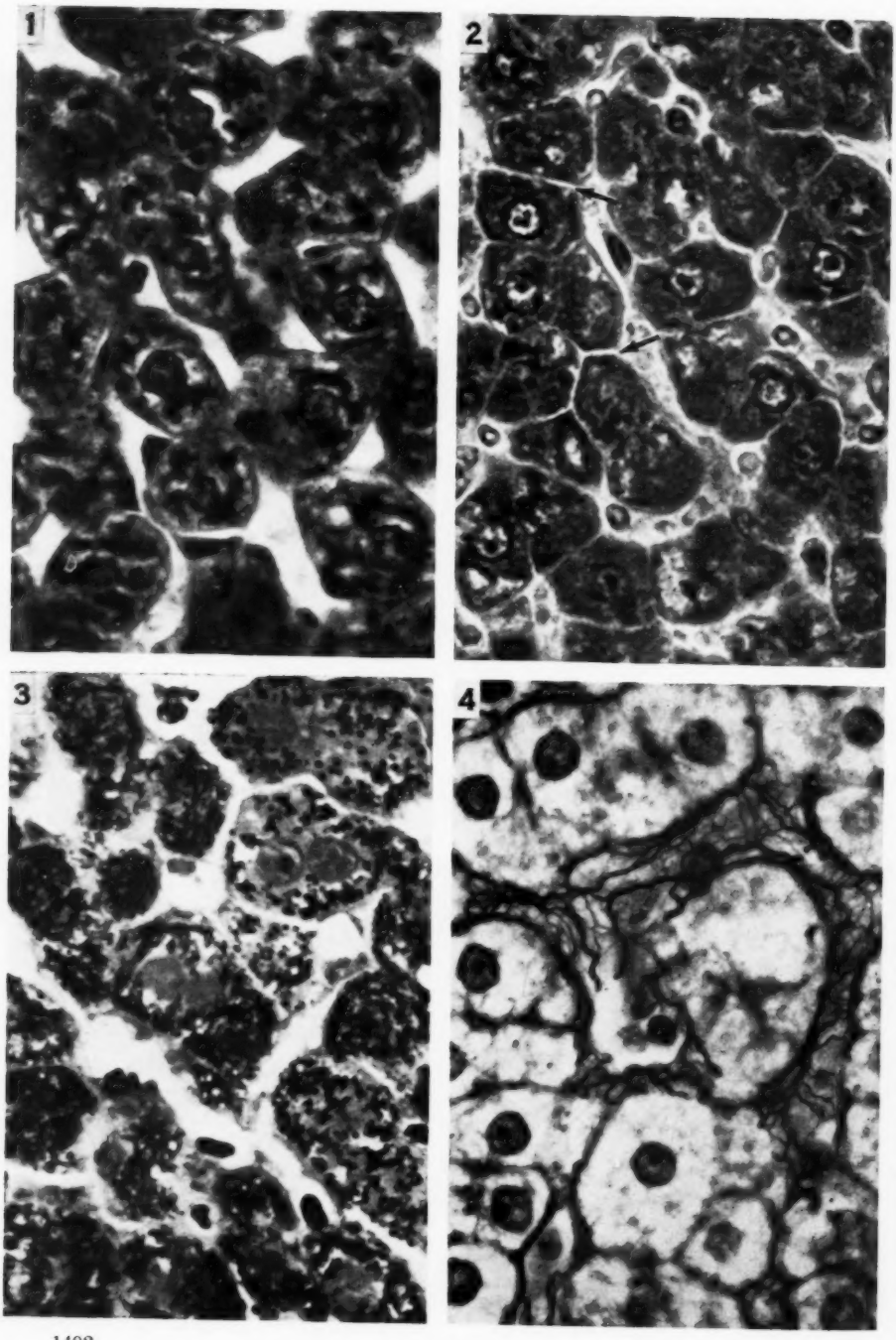
FIGURE 28.—Portions of two adjacent liver cells showing two interlocking cell processes in typical relation to the bile canaliculus. × 16,500

PLATE 114

FIGURE 29.—A section of the surface of a liver cell (*above*) facing a sinusoid (*below*). The cell is covered with slender villous projections or microvilli (mv) similar to those found on free surfaces of many other epithelia. The *lower half* of the figure is occupied by part of a mononuclear leukocyte in the lumen of the sinusoid. At this point the liver cell surface seems to be directly exposed to the blood stream. × 33,000

FIGURE 30.—The margin of this liver cell (*above*) is studded with short villous processes (mv) as was the one in figure 29, but here it is separated from the lumen of the sinusoid by a thin portion of a Kupffer cell which contains a mitochondrion (m) oriented horizontally. The edge of a neutrophilic leukocyte in the sinusoid is seen in the lower part of the figure. × 20,000

FIGURE 31.—Three liver cells from a fasted animal. The cells are less coherent than normal and have been pulled apart in preparation. It is interesting to note that the last points of attachment to give way are at the edges of the bile canaliculi. × 7,000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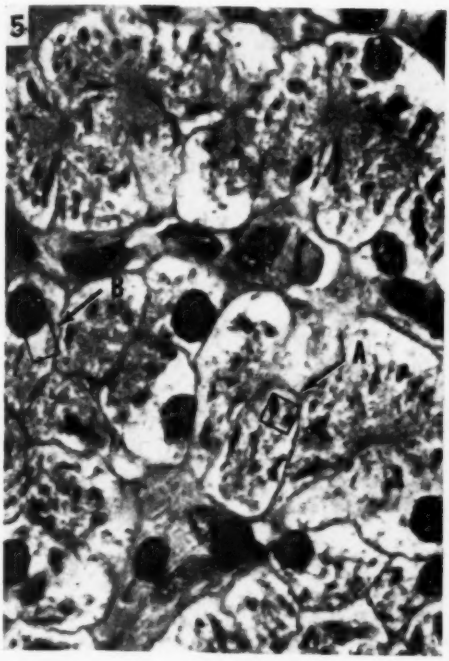
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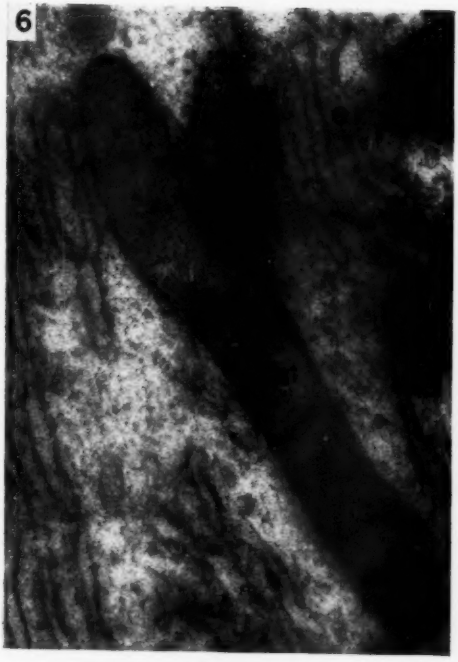
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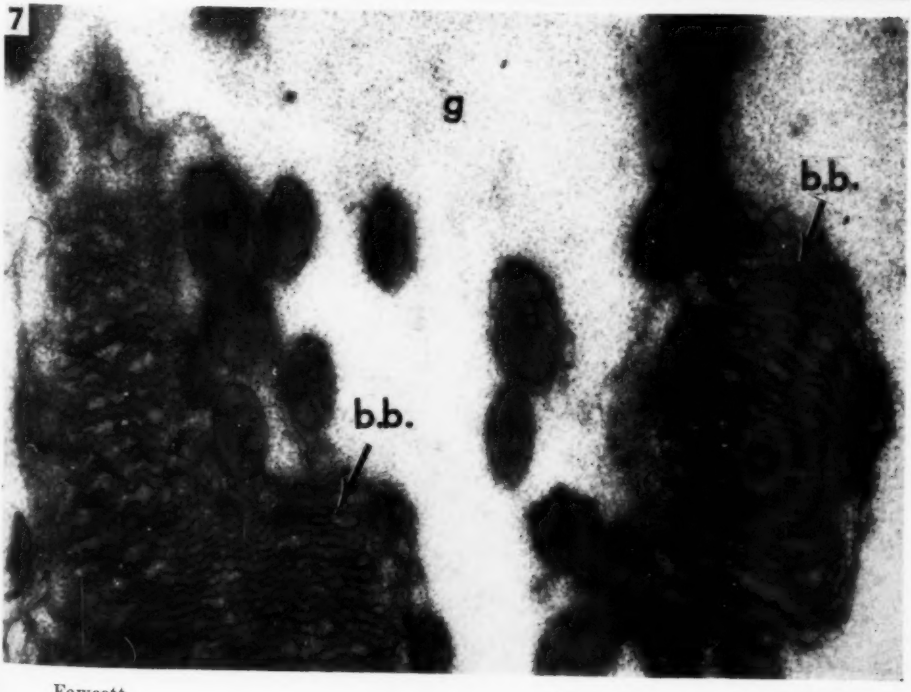
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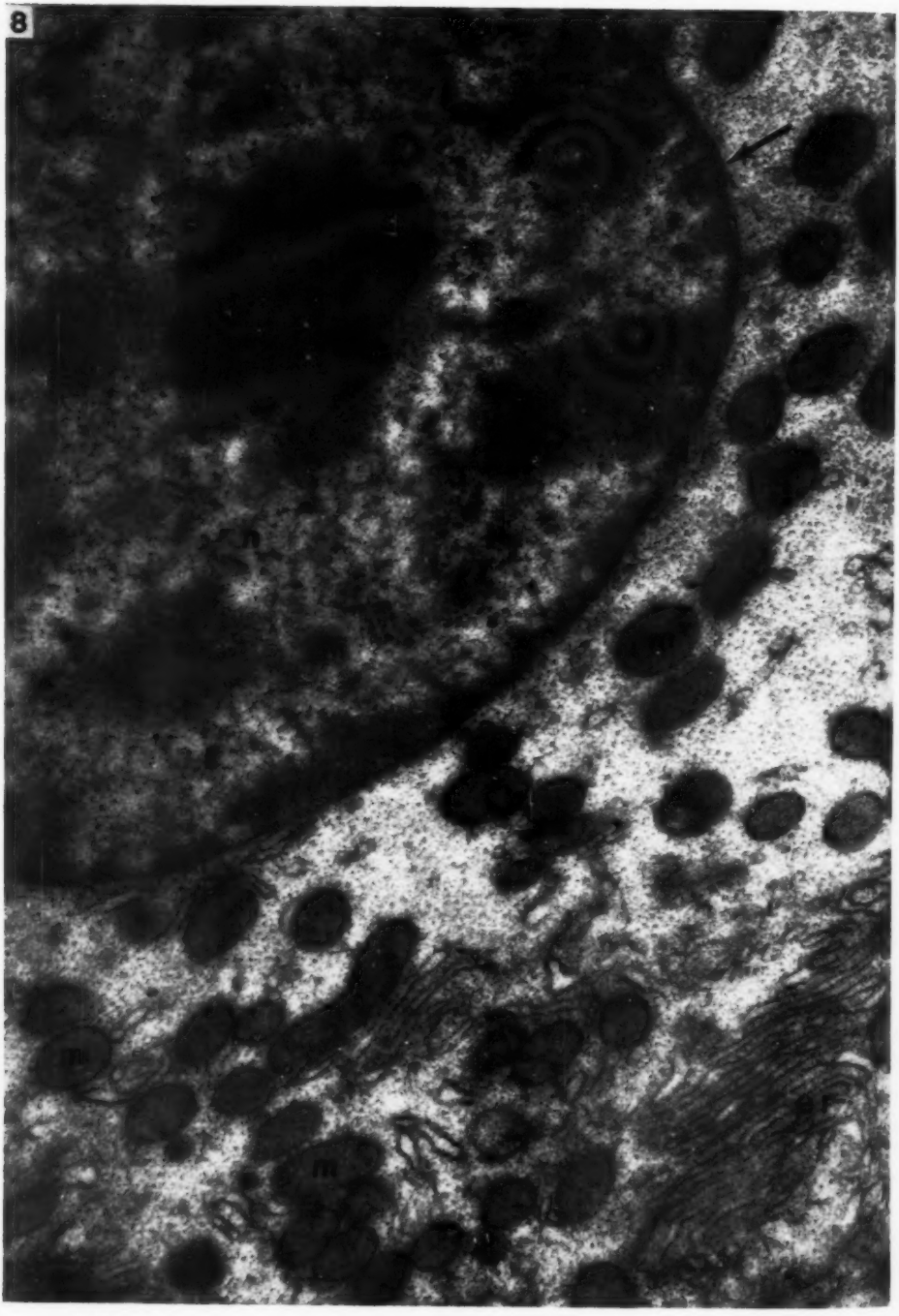
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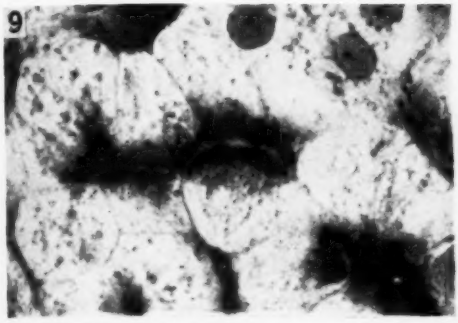

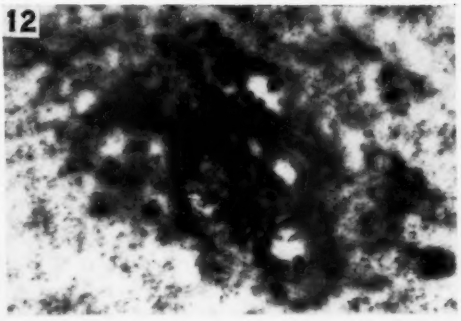

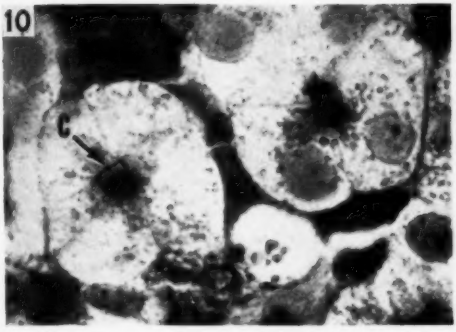

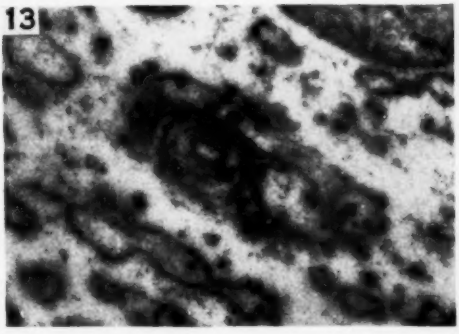

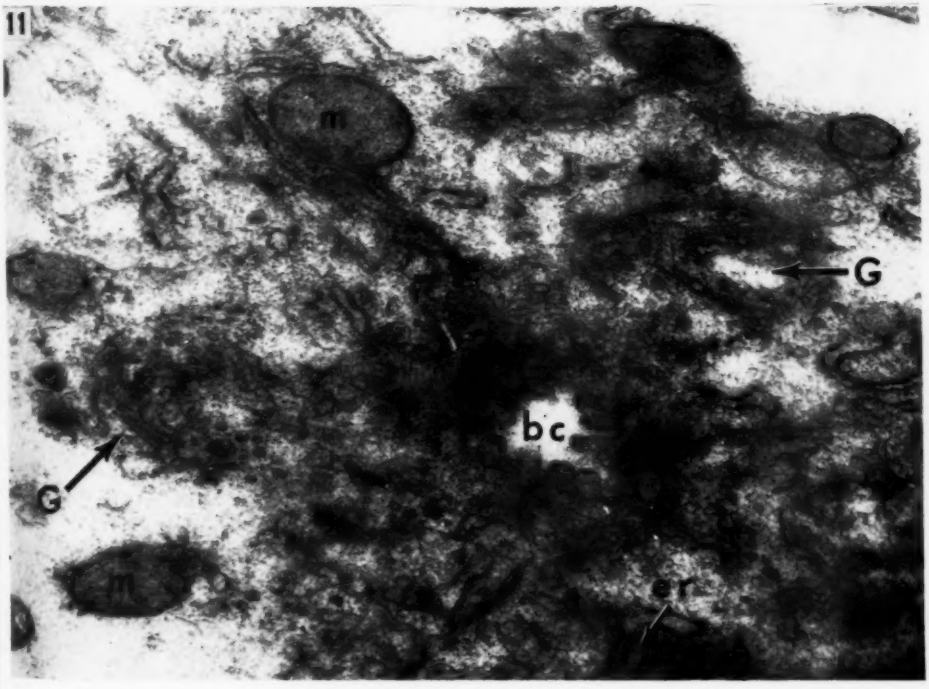

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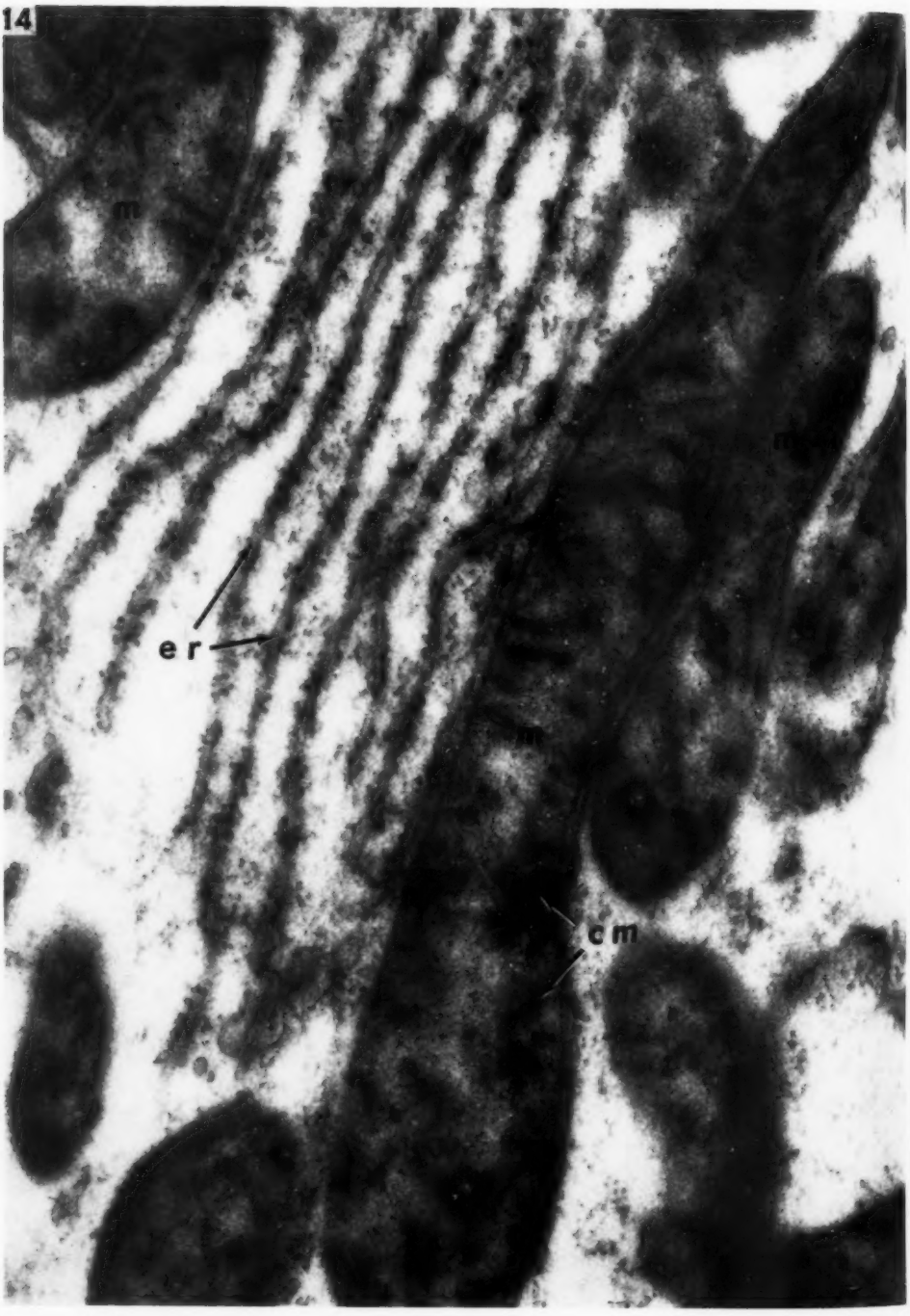
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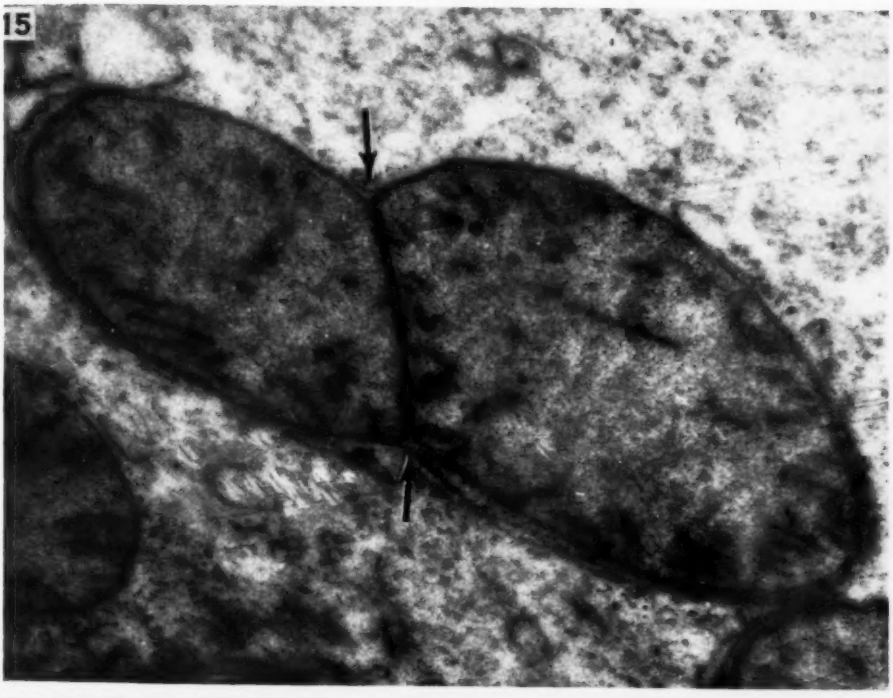
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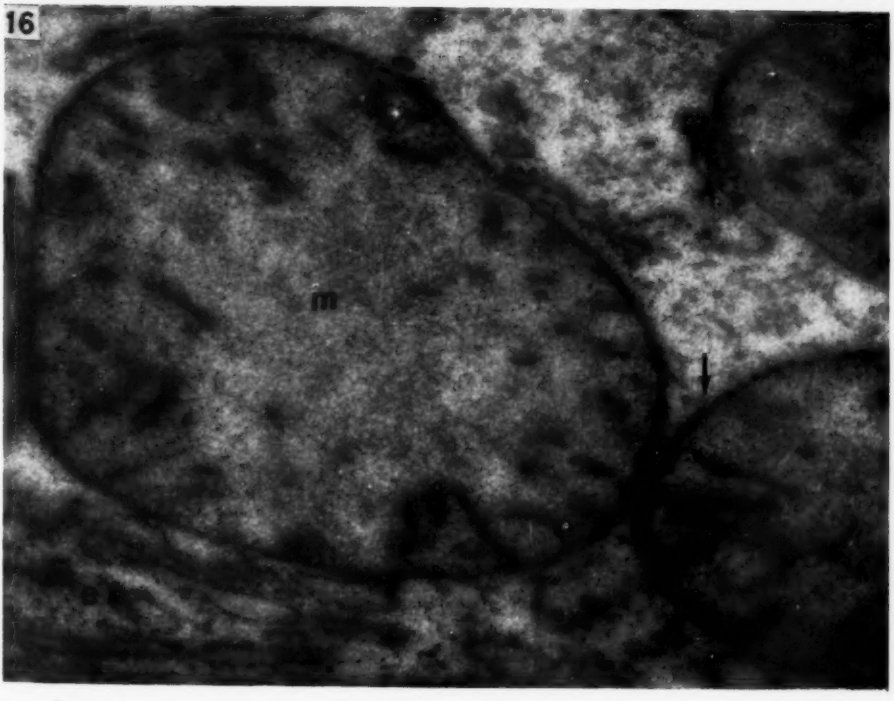
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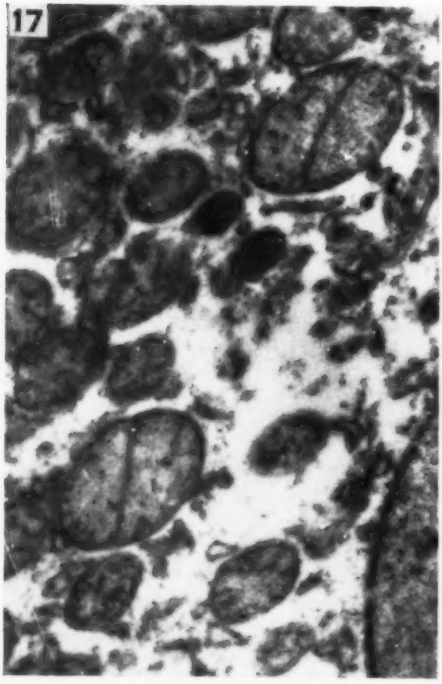
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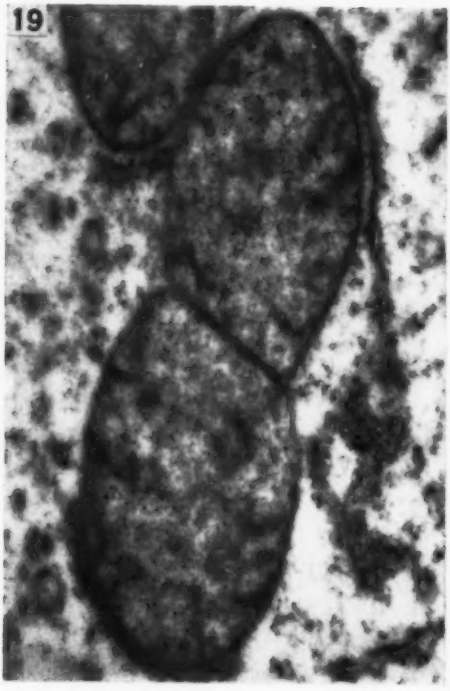
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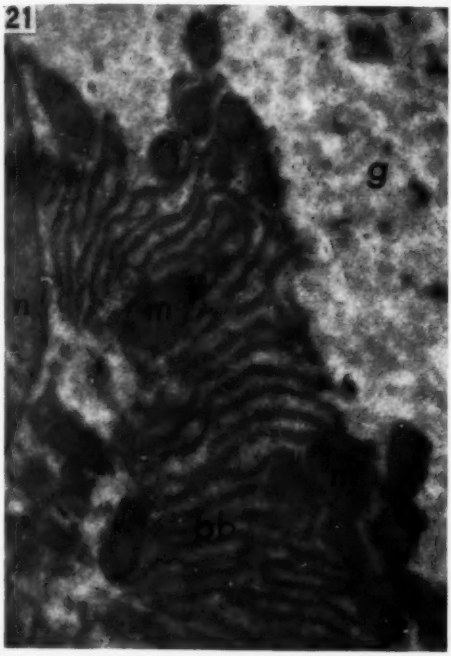
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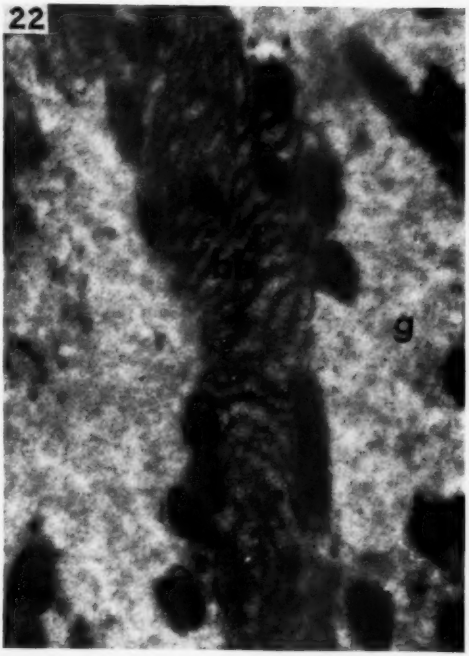
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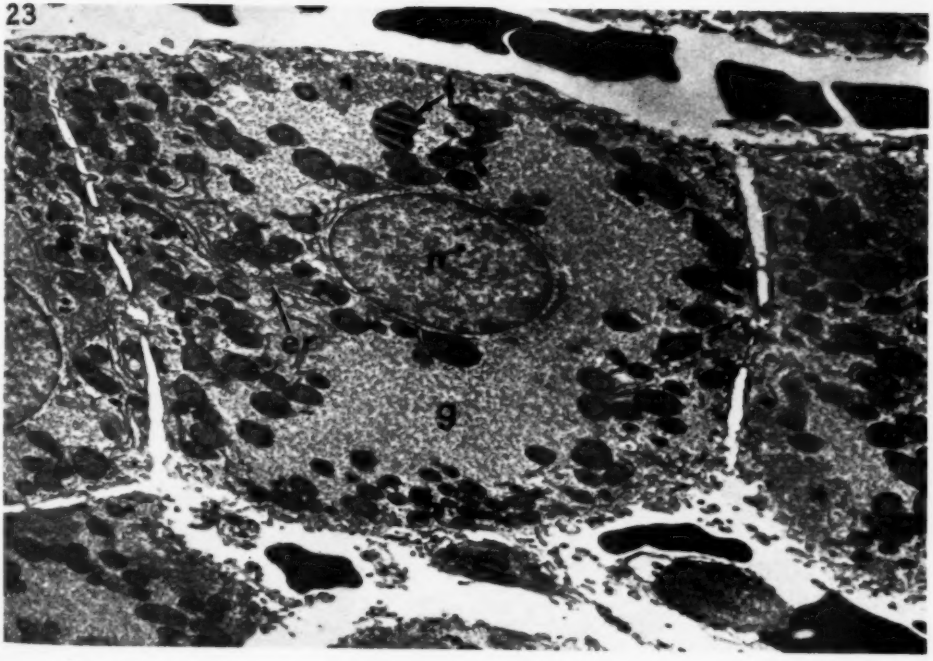
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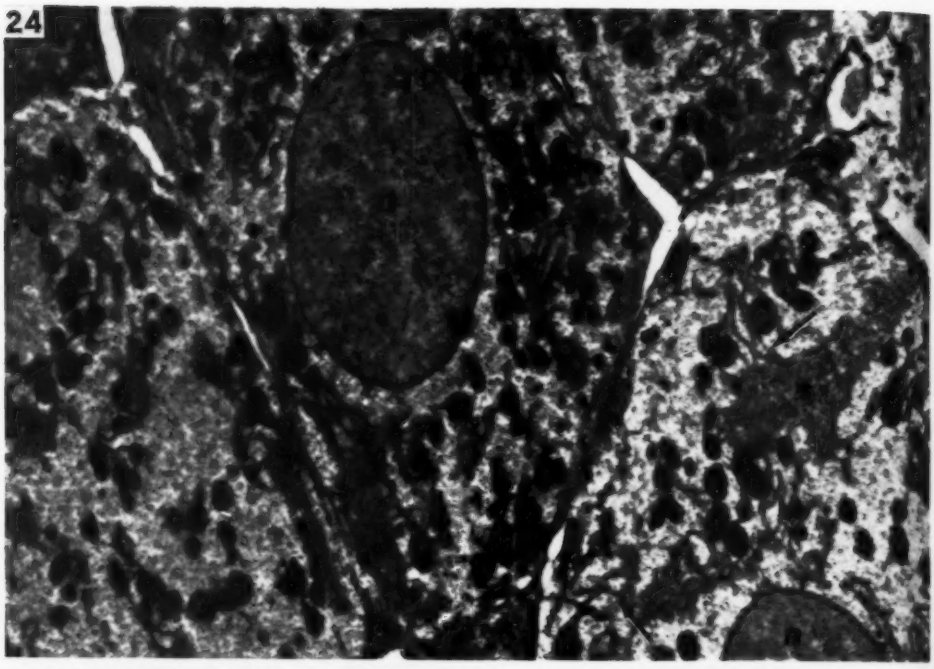

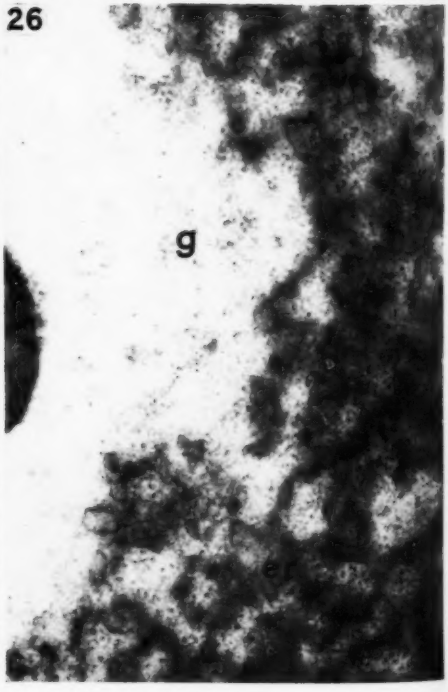

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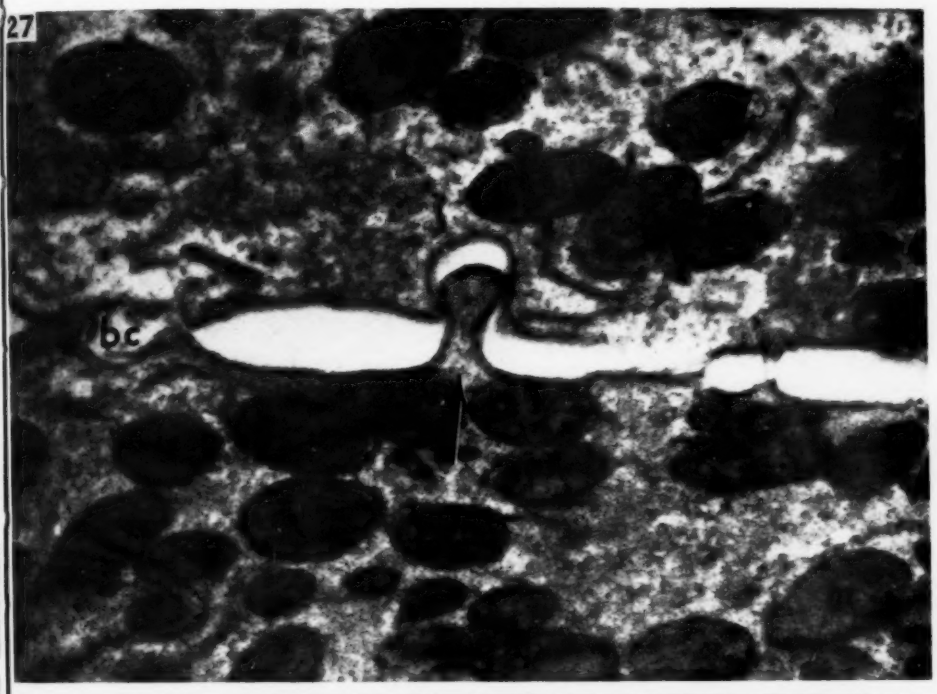
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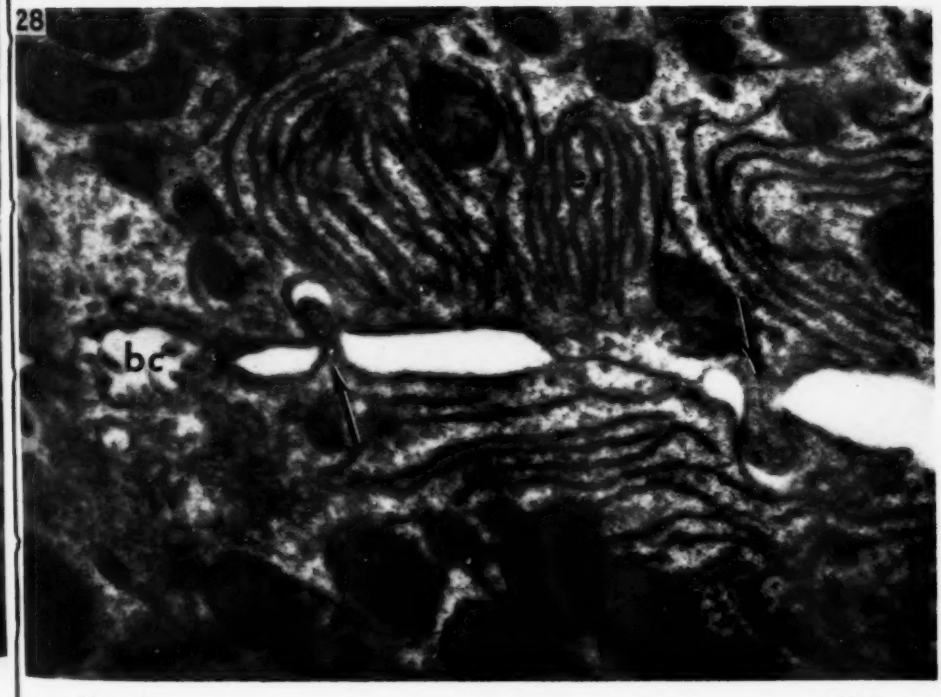
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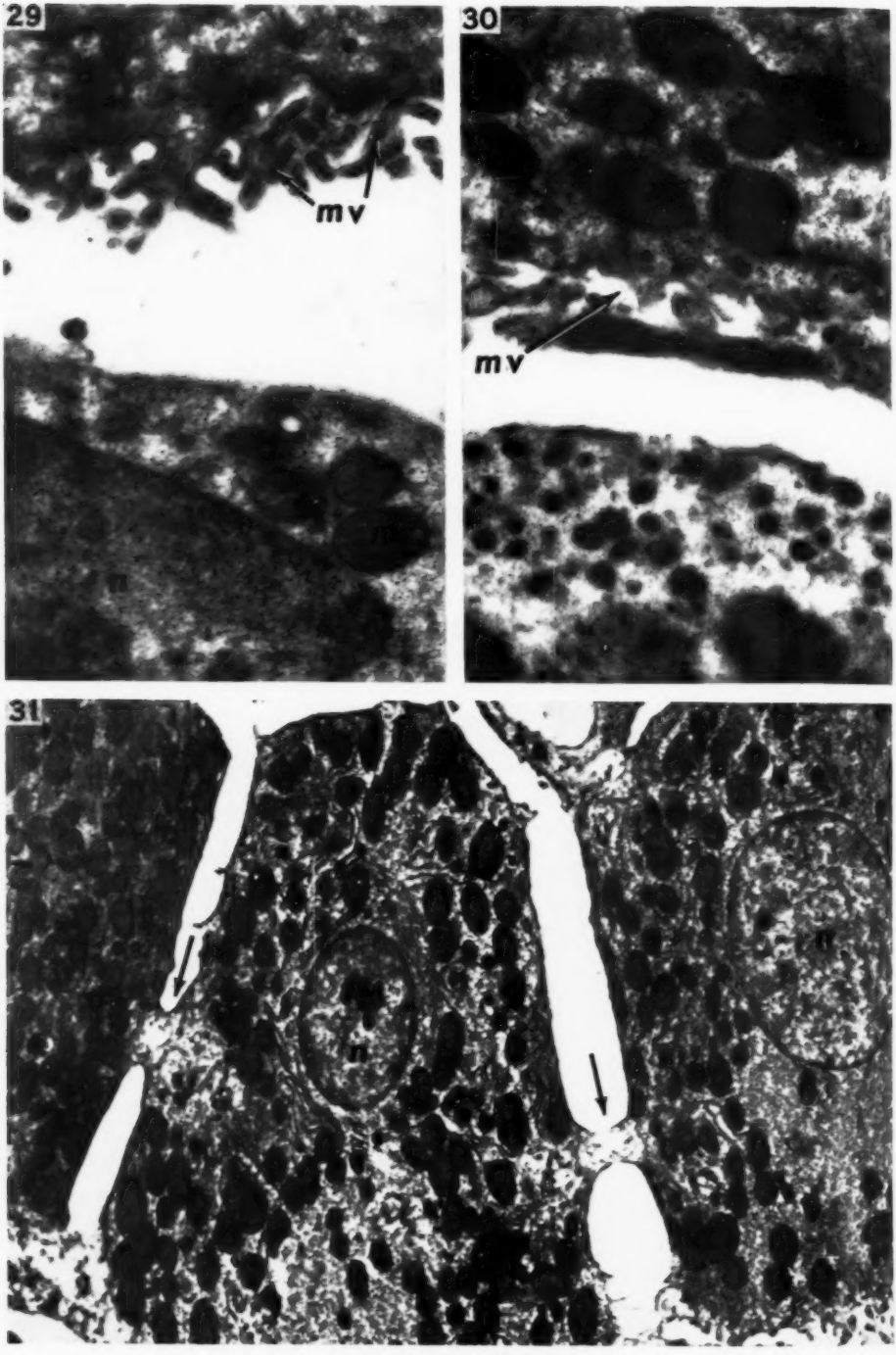
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Dr. Mary L. Petermann, Cornell University Medical College, New York, N. Y.: We have been studying the cytoplasmic nucleoproteins of rat liver and azo-dye induced liver tumors. The livers are perfused with saline, chilled, and homogenized in 0.88 M sucrose, with care not to break nuclei. The tissue debris, unbroken cells, nuclei and mitochondria are centrifuged off at 20,000 × g, and the microsomes and macromolecular nucleoproteins sedimented at 140,000 × g. The pellets are resuspended in *p*H 8.6 buffer and examined in the analytical ultracentrifuge.

In the ultracentrifuge pattern of normal liver there first appears a large, rapidly spreading boundary, caused by the sedimentation of the microsomes (presumably ground-up endoplasmic reticulum) and glycogen. This is followed by four much sharper boundaries, A, B, C, and E, with sedimentation rates of about 70, 50, 40, and 28S, representing the nucleoproteins. These substances correspond in size to the small dense particles, about 200 Å in diameter, described by Dr. Fawcett. Component B predominates, and its concentration shows a strong correlation with the average cell weight (the reciprocal of the number of nuclei per gram). In the livers of rats which were fed *p*-dimethylaminoazobenzene but did not develop tumors, the concentrations of A, B, C, and E were fairly normal. In azo-dye induced hepatomas and cholangiomas, however, the concentrations of C and E were markedly elevated, and the ultracentrifuge patterns resembled those seen in liver samples taken two days after partial hepatectomy, when the rates of growth and cell division were very high. Similar patterns have also been seen in other types of tumors.

The significance of these findings is still unknown. Studies are now in progress on the livers of starved rats, normal and pregnant female rats, etc. We are also attempting to purify and characterize these substances, although they are very unstable. They contain about 45 percent of pentose nucleic acid, all of which is bound to protein.

A Note on the Occurrence of Virus-like Particles in the Spontaneous Hepatomas of C3H Mice[1,2]

DON W. FAWCETT,[3] *and* J. WALTER WILSON,
Department of Anatomy, Harvard Medical School, Boston, Mass., and the Arnold Biological Laboratory, Brown University, Providence, R. I.

In certain sublines of the C3H strain of mice over 90 percent of females develop mammary tumors by the time they are 10 months old and about 25 percent of the males that live longer than 12 months develop spontaneous hepatomas (*1*, *2*). The occurrence of the mammary tumors in females of this strain is known to be influenced by a transmissible agent with many of the characteristics of a virus which is passed from one generation to the next through the milk (*3*, *4*). Virus-like particles, believed to be the milk agent, have been demonstrated with the electron microscope in intact epithelial cells from tissue cultures of spontaneous and transplanted mammary carcinomas of C3H female mice (*5*) and similar bodies have been observed recently with the electron microscope in thin sections of these tumors (*6*). To our knowledge the presence of such particles has not been reported heretofore in the spontaneous hepatomas of C3H male mice.

Observations

Observations were made on thin sections of a spontaneous hepatoma of a male C3H mouse prepared by the methods set forth in a previous paper in this issue (*7*). Some of the hepatoma cells contained dense particles of a sort not normally found in the cytoplasm of liver cells. These particles, which varied from 140 to 170 mμ in diameter, were distributed throughout the cytoplasm but were not found in the nucleus. They were uniformly spherical in shape, and had a dense center and a slightly less dense cortex, but the two regions graded into one another without a sharp demarcation. The particles contained a few extremely osmiophilic granules of small size but no other internal structure could be made out with present resolutions. A few of the particles appeared to have a distinct limiting membrane but this was lacking in the majority (fig. 4). Some were situated within the lumen of tubular elements of the endoplasmic reticulum, and in those instances where there appeared to be a limiting membrane, it is possible that the membrane was not a part of the particle itself but belonged instead to a vesicle of endoplasmic reticulum in which the particle chanced to be enclosed. The number of particles per cell varied from a few to very large numbers which nearly filled the cytoplasm. The presumed viral agent apparently does not readily pass from one cell to another, for tumor cells heavily laden with particles were often surrounded by cells that contained none at all.

The nonparasitized cells were noteworthy for the abundance of their ergastoplasm or the basophilic component of their cytoplasm (fig. 2), which occurred in the form of large masses of loosely packed sheets or flattened vesicles of endoplasmic reticulum.

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N Y., October 11, 1954.

[2] This investigation was supported, in part, by a research grant C-2197 (C) from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[3] Studies carried out during the tenure of a Lederle Medical Faculty Award.

These were somewhat more disorderly in their organization than were the basophilic bodies of normal liver, but the extensive paired membranes tended to be more or less parallel in their arrangement. The small granular component (150 Å) of the cytoplasm, which is usually associated with the endoplasmic reticulum (*8*), was very abundant both on the surface of the membranes of the reticulum and free in the cytoplasm. Mitochondria were unusually small but numerous and were generally closely applied to the periphery of the masses of endoplasmic reticulum (fig. 2). Small clear areas of cytoplasm rich in glycogen were interspersed with the large aggregations of reticulum (fig. 2). The contact surfaces of the hepatoma cells were often covered by numerous slender processes like those found on the free surface of normal liver cells or like those which project into the lumen of the bile capillaries. The villous surfaces of adjacent cells interdigitated in a complicated manner, but the cells did not appear to be more firmly attached to one another than normal liver cells. Typical bile capillaries were not found.

In hepatoma cells containing a moderate number of virus-like particles, the masses of basophilic material were less prominent than in nonparasitized cells (fig. 1), and it was not uncommon for the double membranes of the endoplasmic reticulum to be arranged in spiral whorls around a few virus particles instead of in their usual parallel array (figs. 5 and 6). Such a concentric arrangement was rarely, if ever, seen in normal liver or in hepatoma cells free of particles. In certain areas of the cytoplasm the endoplasmic reticulum took the form of tight reticular masses of small tubules and vesicles (fig. 5) which closely resembled those seen in early stages of the regeneration of cytoplasmic basophilia which occurs upon refeeding after a period of fasting (*7*).

In cells heavily parasitized, conspicuous aggregations of basophilic substance were usually lacking but numerous individual strands of endoplasmic reticulum were still present (fig. 3). Although the amount and state of aggregation of the endoplasmic reticulum in the hepatoma cells was markedly affected by the presence of the virus-like particles, the mitochondria were not noticeably altered. The inverse relationship between the amount of cytoplasmic basophilic substance in the host cell and the number of virus-like particles suggests that the latter may proliferate at the expense of the former. More detailed study of this point may yield information of considerable interest in the interpretation of host-parasite relations.

Discussion

The particulate bodies previously reported by Porter and Thompson (*5*) in epithelial cells from mammary tumors of C3H mice had an average diameter of 132 mμ, while those now described in hepatoma cells were 140 to 170 mμ in diameter. This disparity in size may be attributable to the different methods of specimen preparation used in the two studies. In preparing tissue-culture cells for electron microscopy, they are fixed, then transferred intact to specimen grids and air-dried. It is quite possible that this treatment results in more shrinkage than takes place when cells are gradually dehydrated in increasing concentrations of alcohol, embedded in plastic, and then sectioned as they were in the present study. It is reasonable to believe that the virus-like particles in the mammary tumors and those in the hepatomas are the same and that they are the milk agent. It has been shown by bioassay that the milk agent is present in the liver, as well as in other tissue of C3H mice (*9*, *10*), but at present there is no good evidence that this agent causes the tumors to develop in the liver. Hepatoma cells may simply provide a particularly favorable environment for its proliferation. These points will be clarified by further study of livers from normal C3H mice and of hepatomas from agent-free strains of mice.

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PLATE 115

FIGURE 1.—A field including parts of four cells (A, B, C and D) from a hepatoma. The two cells in the center of the figure (B and C) contain large numbers of dense spherical particles, while the cells at the *left* and *right* (A and D) are entirely free of such particles. × 9,279

FIGURE 2.—Portions of three hepatoma cells which are free from virus-like particles. The endoplasmic reticulum (er) or basophilic component of the cytoplasm is very abundant and somewhat more disordered in its arrangement than in a normal liver cell. Mitochondria are closely associated with the basophilic substance and less common in the areas occupied by glycogen (g). × 11,753

PLATE 116

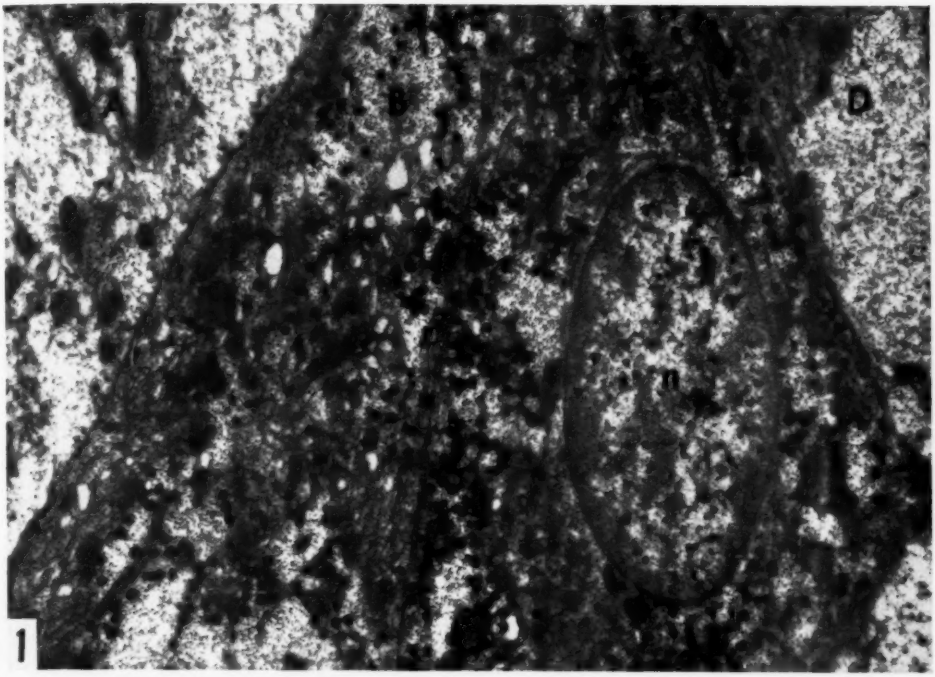
FIGURE 3.—A part of the nucleus (n) and adjacent cytoplasm of a cell very heavily infested with virus-like particles 145 to 170 mμ in diameter. Numerous strands of endoplasmic reticulum (er) pursue a meandering course through the cytoplasm. The mitochondria are unusually small but otherwise normal in their shape and internal structure. Notice *at the arrow* a porelike opening (500 Å) in the nuclear membrane. × 28,600

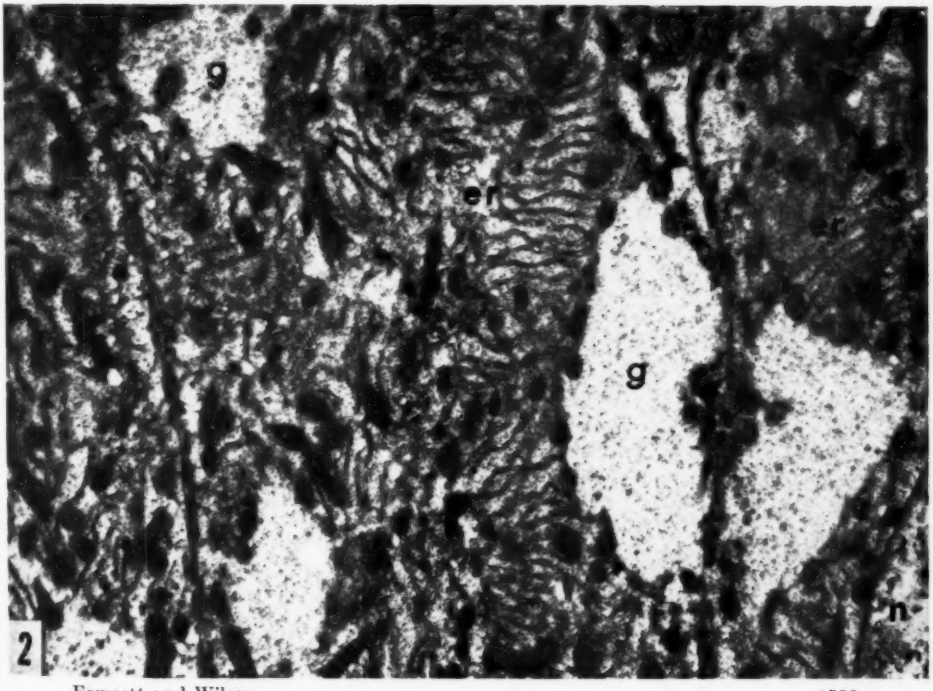
FIGURE 4.—Several spherical particles at high magnification showing a dense center and a slightly less dense cortex. Some seem to have a limiting membrane (*see at arrows*); others do not. A few extremely dense granules can be seen within the virus-like particles. × 135,800

PLATE 117

FIGURE 5.—Area of cytoplasm from a hepatoma cell containing a moderate number of virus-like particles. At the *left* is a basophilic body in which the double membranes of the endoplasmic reticulum have a concentric arrangement and are richly provided with small dense granules. At the *right* is a reticular mass of small membrane-bounded tubules and vesicles which have relatively little of the small granular component. This is believed to be an area of newly formed endoplasmic reticulum. × 33,150

FIGURE 6.—A mass of the basophilic substance of the cytoplasm wherein the double membranes are arranged concentrically around one or two virus-like particles. Such an arrangement of the endoplasmic reticulum is rarely, if ever, seen in normal liver. The small (150 Å) granules closely applied to the surface of the membranes are clearly visible at this magnification. × 69,400





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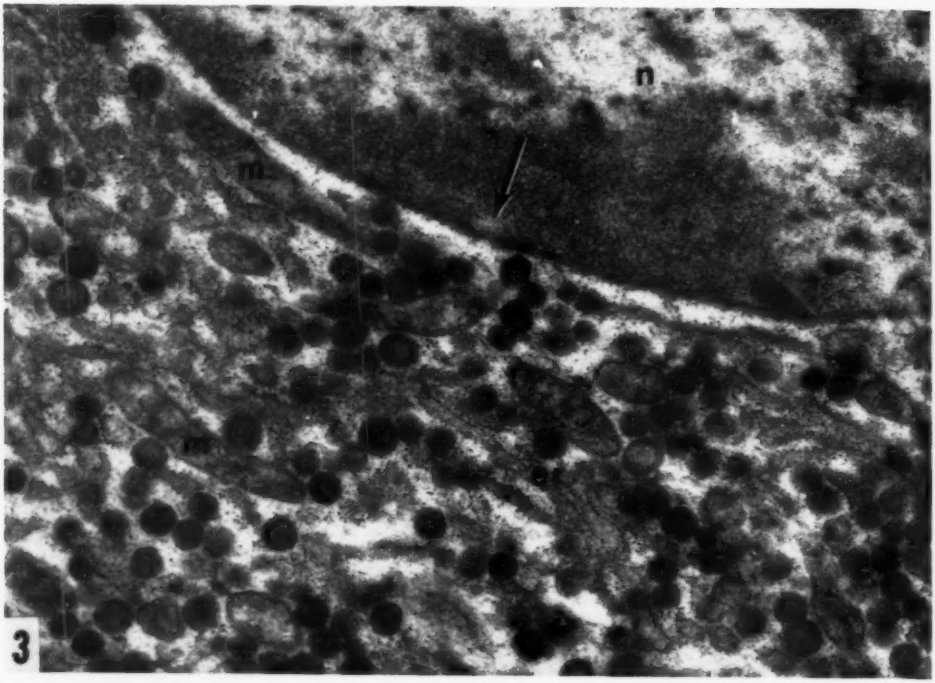
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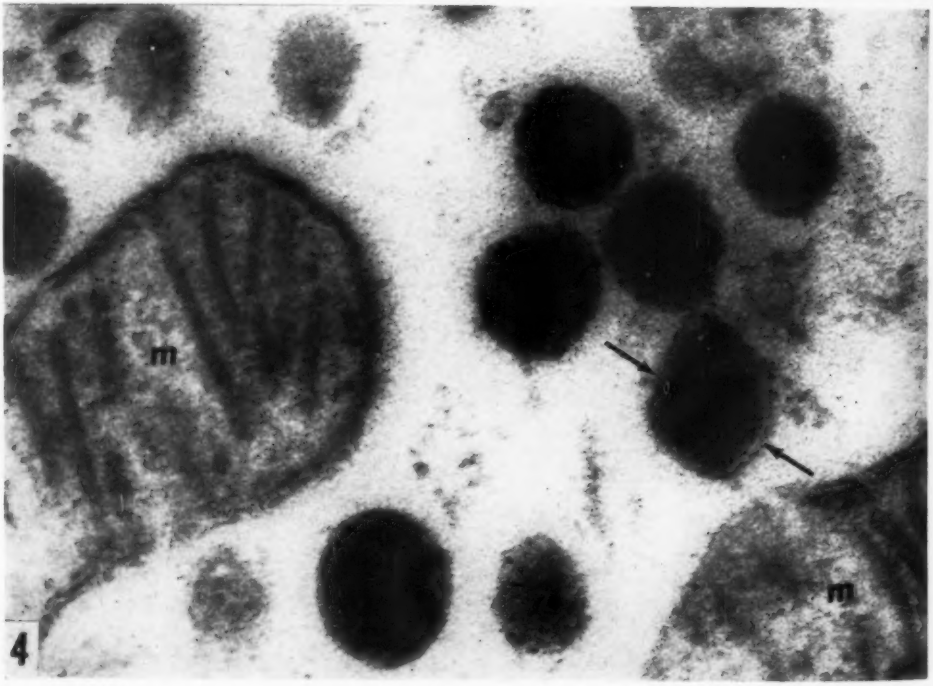
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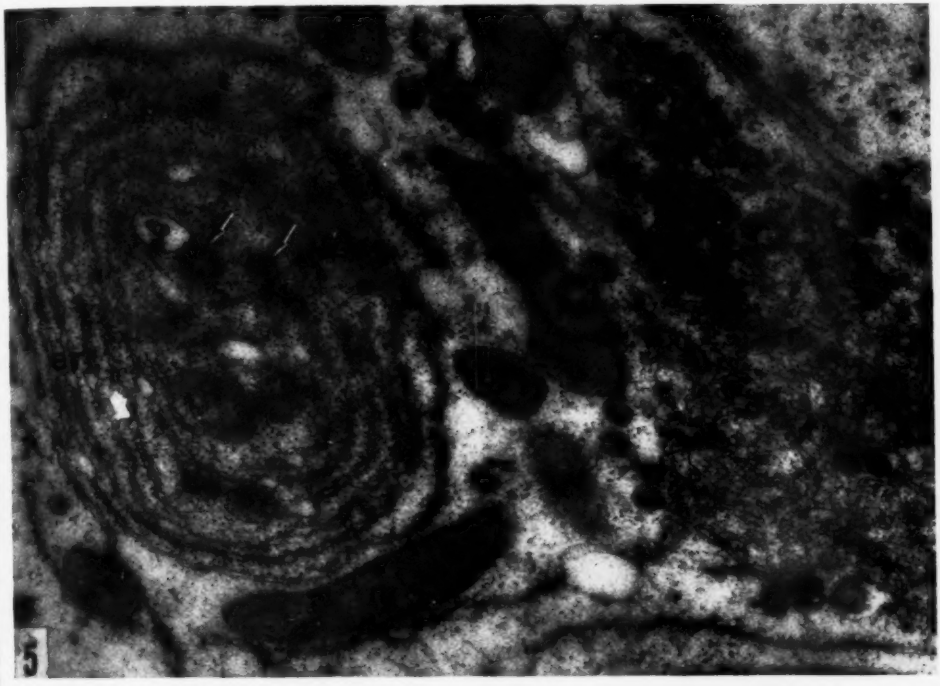

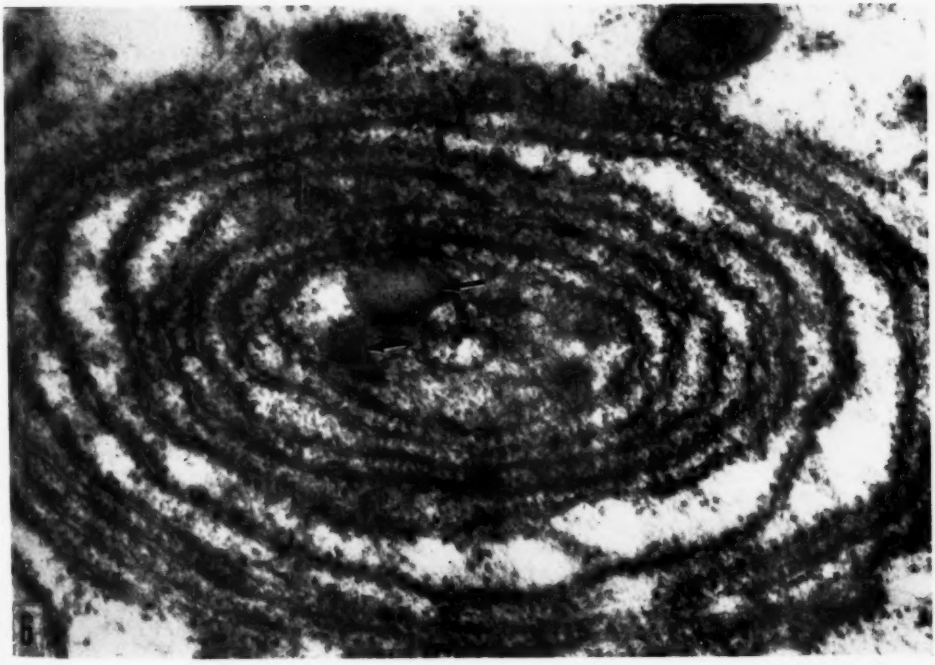

Transplantation of Hepatomas in Mice[1]

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In an earlier study 81 spontaneous or induced hepatomas arising in inbred mice were transplanted subcutaneously into 1 to 4 members of their strains of origin (*1*). Thirty-six of these hepatomas grew in first-generation hosts. Thirty-one were propagated through 2 to 15 transplant generations and then discontinued, and 5 failed to grow after 1 to 6 transplant generations. Most of the successfully propagated hepatomas failed to grow in all recipients. This, together with the inability of 45 primary tumors to become established in new hosts and the inability of 5 hepatomas to undergo serial passages, indicates that nongenetic factors were of importance in the transplantability of these hepatomas.

Of 51 primary hepatomas induced with *o*-aminoazotoluene, 23 grew in new hosts, whereas of 8 hepatomas induced with carbon tetrachloride none grew. This finding suggested that hepatomas induced with carbon tetrachloride may be more difficult to transplant successfully than those induced with *o*-aminoazotoluene. Of 19 primary spontaneous hepatomas 13 became established in new hosts.

Six hepatomas induced with *o*-aminoazotoluene were transformed to hemangioendotheliomas while undergoing transplantation and 4 spontaneous hepatomas were transformed to tumors of 4 different types. These transformations were described in the earlier publication (*1*). A change to hemangioendothelioma in strain C mice usually occurred abruptly and was found after from 1 to 8 passages. Hepatoma No. 10 was of particular interest because sublines developed, some of which transformed while others remained hepatoma. This line also showed, in the second generation, an area of hemangioendothelioma at the margin of the hepatoma, but complete transformation did not develop until a later generation.

Transformations developing from spontaneous hepatomas in strain C3H resulted in 4 different types of tumor diagnosed as 1) osteogenic sarcoma, 2) reticulum-cell sarcoma, 3) a possible cholangioma, derived from bile-duct epithelium, and 4) an adenocarcinoma in which the tissue of origin was undetermined. The osteogenic sarcoma and the "cholangioma" types of growth that developed during serial transplantation of a hepatoma appeared abruptly from one generation transplant to the next. The change to reticulum-cell sarcoma in No. 31 took place through several generations. In No. 32 areas of hepatoma were still found, although the bulk of the tumor was adenocarcinoma. This tumor was discontinued because it was suspected of being a mammary tumor. The change in hepatoma No. 30, the questionable cholangioma, is discussed later in connection with the present experiment.

Histologically, a primary hepatoma of the mouse resembles normal liver very closely (fig. 1). The normal liver surrounding the hepatoma is often compressed by the expanding neoplasm. In some areas the hepatoma cells are sometimes smaller than

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the normal hepatic cell. This is an unusual feature in a neoplasm, in which increased size of the cells is often emphasized as a criterion of malignancy. Adenocarcinoma, in comparison, is a very rare form of primary liver neoplasm in the mouse. One was induced in a strain C57BR by feeding *p*-dimethylaminobenzene-1-azo-2-naphthalene (*2*) (fig. 2). During serial transplantation of hepatomas, as is discussed later, development of adenocarcinoma is not so unusual.

The results just described were obtained with induced tumors in mice of strains C and I and with spontaneous tumors in mice of strain C3H. In order to ascertain whether histologic study could reveal any qualitative difference between induced and spontaneous hepatomas, it was decided to procure both types of tumors from mice of the same strain. Strain C3H animals were used because of the relatively high incidence of spontaneous hepatomas in them.

o-Aminoazotoluene and carbon tetrachloride were used to produce hepatomas. *o*-Aminoazotoluene was chosen because mice of strain C show a pronounced sex difference in susceptibility to induced hepatomas following subcutaneous administration of the compound (*3*). Females are more susceptible than males. Since strain C3H males are more susceptible than females to the development of spontaneous hepatomas, it was decided to ascertain whether in this strain this azo dye also elicits more tumors in females (*4*).

Carbon tetrachloride was administered because in the previous experiment 8 primary tumors induced with this compound in mice of strain I failed to grow in new hosts. It was hoped that transplantation of tumors induced with both compounds into mice of the same strain would reveal whether those produced with carbon tetrachloride were more difficult to transplant successfully than those induced with *o*-aminoazotoluene. It was also desirable to learn whether strain C3H animals show a sex difference in susceptibility to hepatomas induced with carbon tetrachloride.

During the course of the experiment a number of hepatomas were transplanted and some of the results attending this part of the experiment are presented. Since some of the tumors are still undergoing serial passage the only findings presented in any detail are those in which the tumors became transformed histologically or were of unusual interest.

Experimental Procedure

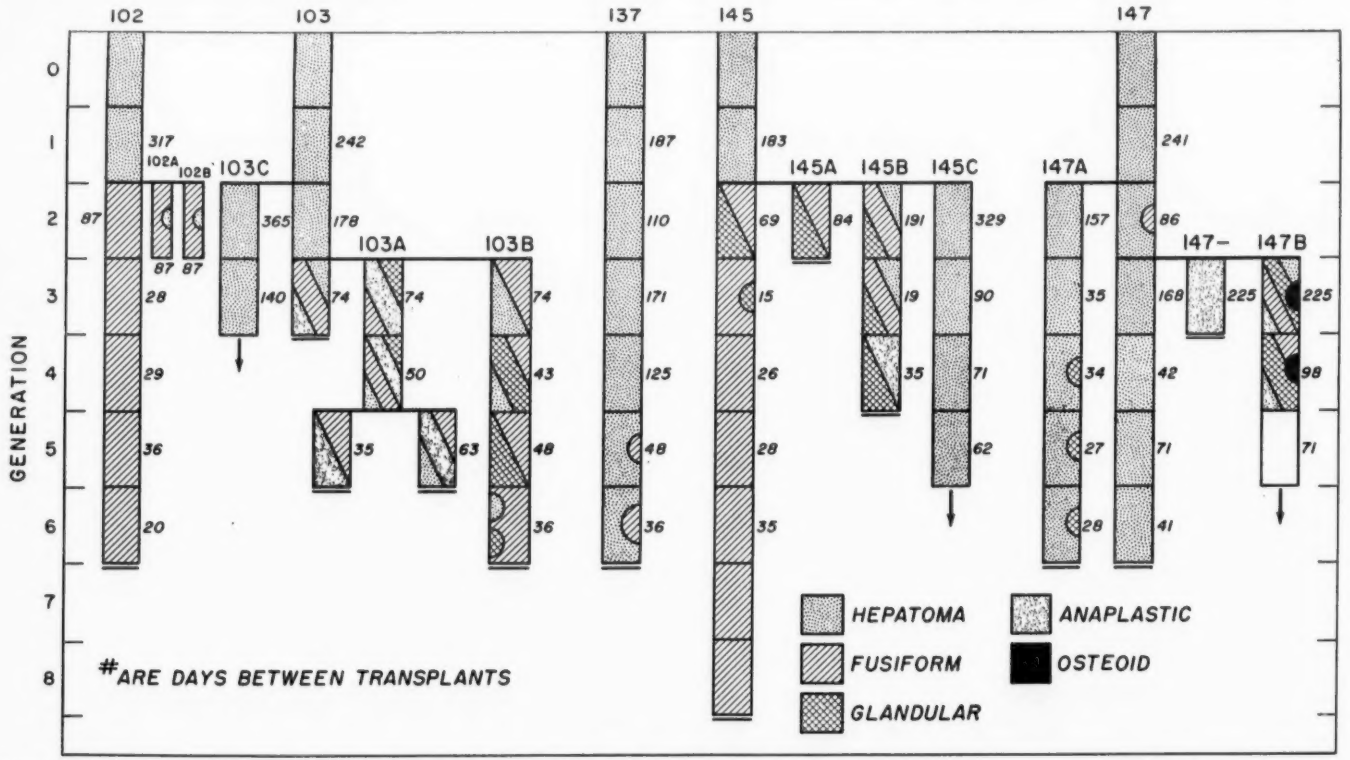
Strain C3H mice of both sexes and between the ages of 3 and 6 months were used. Some carried and others were free of the mammary tumor agent. Litter mates were divided equally and according to sex between those receiving the azo dye and those receiving carbon tetrachloride. Those given *o*-aminoazotoluene each received, at monthly intervals, 4 subcutaneous injections of 10 mg. of the compound dissolved in 0.5 cc. of olive oil, for a total of 40 mg. Carbon tetrachloride was dissolved in olive oil and administered by means of a stomach tube. The first dose of 0.25 cc. of a 4-percent solution of carbon tetrachloride was toxic and killed a number of mice. One week later 0.15 cc. of the same concentration also proved to be toxic. In the following 2 weeks, the dose was lowered to 0.2 cc. of a 2-percent solution which was tolerated by all the mice. Each mouse then received, at weekly intervals, 17 feedings of 0.2 cc. of a 3-percent solution. Few deaths resulted from the administration of this dose of carbon tetrachloride.

Spontaneous hepatomas were obtained from untreated males when they were 15 months of age.

The mice were killed when 10 to 16 months old. A portion of the hepatoma selected for transplantation was prepared for histologic study and pieces of the remainder implanted subcutaneously by means of the trocar technique.

Forty-six hepatomas were transplanted. Of 10 spontaneous hepatomas, 9 underwent 6 serial passages while 1 failed to grow in the first transplant generation. Of 30 from carbon-tetrachloride-treated mice, 2 failed to grow when transplanted, and of 6 from *o*-aminoazotoluene-treated mice, 1 failed to grow in serial transfer. Thus, of 46 transplanted hepatomas, all but 4 were carried through 4 to 6 transplant generations.

We shall report briefly on 7 of these hepatomas. The first 4 occurred in mice that had received carbon tetrachloride. Text-figure 1 shows the complex morphologic



TEXT-FIGURE 1.—Chart showing generations of transplanted liver tumors in the mouse. Number of generation shown on *left*. 0 represents primary tumor in the liver. Number of tumor shown at *top*, and number of days from time transplant was made until the animal was killed or died are given to the *right* of each generation. Different types of histologic structure developing in the transplanted tumors are shown schematically. *Arrows* indicate that tumor is still being carried.

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changes which developed in these hepatoma lines during transplantation. In this experiment, we were given a better opportunity to observe morphologic alterations than in the earlier experiment, since the changes did not always occur abruptly from one generation to the next. Five types of tissue which were morphologically distinguishable are represented in the chart. These are: 1) hepatoma; 2) tissue composed of fusiform cells, usually indistinguishable from fibrosarcoma; 3) tissue having a glandular pattern; 4) an anaplastic tumor tissue, often resembling reticulum-cell sarcoma; and 5) small patches of tissue with unmistakable bone formation. This chart is highly schematic and the changes in the transplanted tumors are even more complex than shown here. Serial sections would be required to show all the morphologic variations occurring in each tumor.

The first hepatoma (No. 102) occurred in a 12-month-old, agent-free male. It measured 15 × 11 × 8 mm. and was the only hepatoma in this mouse. A piece was prepared for histologic study and the adjacent portion used to inoculate 5 male animals. It grew in only one mouse of the first transplant generation and required 317 days to reach a size of 11 × 10 × 7 mm. This first generation tumor resembled liver tissue grossly and was transplanted into 4 males. Growth occurred in 3 animals and one tumor was transplanted 87 days after inoculation when its gross appearance was described as "white and firm." This tumor was carried through 4 more passages in a total of 13 mice and grew in every recipient. The last passage required only 20 days to become 15 × 10 × 7 mm. in size.

The primary tumor was treated by the periodic acid-Schiff technique to reveal glycogen. This procedure is very useful in identifying liver tumors. In normal liver, the glycogen may be scarce or abundant, but it is always uniformly distributed in relation to the lobular pattern. In hepatomas, on the other hand, the glycogen may be abundant or absent in many parts of the tumor, but there is no uniform distribution and the amount usually shows a marked variation from that in adjacent normal liver (fig. 3).

The first generation transplant of this tumor was still recognizable as a primary liver-cell tumor (fig. 4). Glycogen was irregularly distributed in the tumor.

During serial transplantation a histologic transformation occurred in this tumor to a type of tumor tissue indistinguishable from a fibrosarcoma. This transformation was complete in the second generation of the main line (fig. 5). All evidence of glycogen disappeared following the transformation to sarcoma.

Sections from two tumors which developed following transplantation from the first generation were predominantly fibrosarcomatous, although a few islands of hepatoma tissue persisted (text-fig. 1). These tumors were not transplanted.

The second hepatoma (No. 103) arose in a 14-month-old male carrying the mammary tumor agent. It measured 31 × 25 × 13 mm. and was the only hepatoma in this animal. Pieces were inoculated into 3 males but only one grew, and it measured 14 × 12 × 8 mm. 242 days after implantation. This first generation tumor was inoculated into males, 3 of which developed tumors. One was killed 178 days following inoculation. The tumor in this mouse measured 8 × 6 × 4 mm. and was described as "cystic but resembling liver tissue." Pieces of this second generation tumor were transplanted into 2 C3H males for the third passage. They grew in both hosts, and 74 days later one tumor was used for passage. This tumor measured 19 × 13 × 7 mm. and was of interest because it was, in part, "firm and white" and, in part, "soft and hemorrhagic." Each part was used to inoculate 2 male mice as fourth transplant generation hosts.

A variety of morphologic changes developed in this tumor (text-fig. 1). The original tumor closely resembled normal liver and other hepatomas of the mouse. In the fourth generation of 103A, an adenocarcinomatous pattern was mingled with an anaplastic tissue composed of large detached mononuclear cells (fig. 6). In the fifth generation there were fusiform cells with collagenous fibers, and also areas of anaplastic tissue.

The fourth generation transplant of tumor line 103B reproduced the pattern of the adenocarcinoma in the first experiment in which the tissue of origin was undetermined.

Periodic acid-Schiff treatment showed foci of cells containing glycogen. These cells resembled hepatic cells. Other areas showed a glandular pattern with rows of cuboidal cells having no resemblance to hepatic cells (fig. 7). This line, 103B, appeared to be transforming to pure fibrosarcoma when it was discontinued.

The third hepatoma (No. 137) represents another transformation. It arose in a 12-month-old, agent-free female. The liver of this mouse contained 2 hepatomas. The largest measured 18 × 17 × 6 mm. and was used to inoculate 4 females and 6 males. It grew in females only of the first transplant generation. After 187 days the largest tumor was used to inoculate 2 males for the second transplant generation and grew in both mice. It grew in all of the 11 males used for the next 4 serial transfers. During the first 4 passages this tumor required from 110 to 187 days to attain a size of 15 × 15 × 10 mm. and was described grossly as "soft, cystic and hemorrhagic." The fifth generation transplant was grossly white and cystic and measured 22 × 19 × 14 mm. 48 days after implantation. The sixth transfer generation resembled the fifth transplant generation.

Histologically, the fifth and sixth passages showed areas of the tumor composed of both fusiform cells and cells resembling those of the primary hepatomas.

The fourth hepatoma (No. 145) arose in an 11-month-old, mammary tumor agent-free male. The liver of this animal contained 2 hepatomas. One measured 23 × 16 × 8 mm. and was transplanted into 5 males. It grew in only 1 of the first generation hosts and measured 16 × 13 × 8 mm. after a growth period of 183 days. This first generation transplant had the gross appearance of liver tissue. It was transplanted into 4 mice of the second transplant generation. When again transplanted, 69 days later, it was firm and white. This line was carried through 6 passages and maintained the same gross appearance throughout transplantation periods of 15 to 35 days.

Another line of this tumor was established from a second transplant generation animal 329 days after implantation. It is in the sixth transfer generation and has not become transformed.

Histologic examination of tumors from the two sublines showed that one remained pure hepatoma, and the other was transformed to fibrosarcoma. The hepatoma line is still being carried and may yet show an alteration.

One tumor from the transformed line showed a mixture of a fusiform and a glandular pattern (fig. 8). One area resembled the questionable cholangioma of the first experiment. Another tumor from this transplant series (145B—Generation 2) is of interest because one area showed an intimate blending of epithelial and fusiform cells (fig. 9).

The fifth hepatoma (No. 147) arose in a 16-month-old, mammary tumor agent-free female that had received azo dye. It was the only hepatoma in this animal and was 8 mm. in diameter. Pieces were implanted into 3 females, all of which developed tumors. One was used for passage after a period of 241 days.

Three lines of transplanted tumors were established. Two were started from mice of the second transplant generation. One of these was maintained for 6 generations, the last generation requiring a transplant period of 41 days. It retained the gross appearance of liver at all times. The other established a growth period of from 27 to 35 days within 6 generations and was consistently described as being soft and white. The third line was started from a third transfer generation in which it measured 11 × 8 × 6 mm. 225 days after inoculation. This tumor was firm and white and retained the same gross appearance for 2 more generations.

Text-figure 1 shows the complex histologic changes in tumor 147. Small areas of a different type of tissue developed in 2 sublines. Subline B is still being carried.

A very complex mixture of tissue developed in the third generation of 147B (fig. 10). Some persisting hepatoma tissue contained glycogen. Several masses of osteoid tissue and one area of infiltration with normal-appearing lymphocytes were found (fig. 11).

Osteoid tissue also appeared in the next generation. This line has been transplanted to a number of mice, and we are curious to see whether an unmistakable osteogenic pattern may develop.

The sixth hepatoma (No. 129) was found in a 14-month-old, agent-carrying male that had received carbon tetrachloride. The liver of this mouse contained 3 hepatomas, and 1 measuring 26 × 23 × 12 mm. was used for passage to agent-carrying C3H mice (4 females and 2 males). A male of this first passage generation had a palpable tumor within 60 days. After another 62 days, the tumor from this mouse was 18 × 15 × 8 mm. in size and was used for passage.

This tumor is presented because it was the most rapidly growing tumor of the present series. It grew in all inoculated mice and attained a large size within 2 to 3 weeks. Because of its ability to grow rapidly it has been sent to various laboratories.

In later generations many of the hepatoma cells were detached and of large size (fig. 12). They contained no glycogen. This tumor has been grown in an ascites form, and these unattached cells may represent the cell form which was able to live free in the peritoneal cavity.

In the thirtieth generation this tumor resembled an anaplastic carcinoma, but it has not undergone the radical change in morphology of some other tumors.

The seventh hepatoma (No. 134) changed remarkably in its gross appearance but retained the histologic structure of a hepatoma. It occurred in an 11-month-old, agent-free female that had received carbon tetrachloride. It was transplanted into 4 males and grew in 3 of these first generation hosts, 2 of which supplied tumors for second generation recipients. Both tumors were described grossly as "resembling liver." One was transplanted 284 days following implantation and underwent 2 serial passages in which it resembled liver tissue both grossly and histologically. The other first generation transplant remained in its host for 368 days. It also resembled liver tissue grossly and histologically. This line of the tumor grew rapidly and underwent 5 serial passages. The last 2 required only 21 and 28 days. Tissues from the last 3 passages were described grossly as "firm and white"—a characteristic of most transformed hepatoma tissues. However, upon histologic examination the tumors were diagnosed as hepatomas.

This is our first experience with a pronounced gross change in a transplantable hepatoma which was not accompanied by a similar change in histologic structure.

Histologically, the early generations of this tumor line showed the usual morphology of a hepatoma. A cordlike arrangement appeared in the first generation, but the cells resembled those of hepatoma. In the fifth generation the cells still resembled liver cells, but areas of the tumor showed an increase in fibrous tissue. This tumor appeared to be invading muscle (fig. 13), which is unusual in hepatomas of the mouse. Mitotic figures were abundant in this tumor, and this feature, along with its invasive power, may partly account for the rapid growth rate.

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PLATE 118

FIGURE 1.—Primary hepatoma. Neoplastic tissue on *left* resembles normal liver, but cells are of smaller size. Adjacent normal liver is compressed by tumor. A smaller focus of hepatoma in *lower right*. × 100

FIGURE 2.—Adenocarcinoma of liver in a C57BR mouse fed *p*-dimethylaminobenzene-1-azo-2-naphthalene. Neoplastic tissue with small spaces lined by a single layer of cuboidal epithelium and separated by abundant connective-tissue stroma. Small area of hepatoma type in *lower left*. × 235

PLATE 119

FIGURE 3.—On *left* hepatoma showing irregular distribution of glycogen. It is increased in some cells, absent in others, and does not show the lobular distribution shown in normal liver to *right* of cleft. Periodic acid-Schiff technique. × 65

FIGURE 4.—Tumor 102, first generation. Usual appearance of hepatoma, with close resemblance to normal liver. × 100

FIGURE 5.—Tumor 102, second generation. Tumor has altered and is indistinguishable from fibrosarcoma. Cells are fusiform, and have a fascicular arrangement. × 235

PLATE 120

FIGURE 6.—Tumor 103, fourth generation of subline A. On *left* adenocarcinoma pattern; small spaces are lined by cuboidal epithelial cells. On *right* an anaplastic area; large detached cells, varying in size, and often having eccentric nuclei. × 235

FIGURE 7.—Tumor 103, fourth generation of subline B. In *lower right* cells resembling hepatoma contain masses of glycogen. Remaining area shows an adenocarcinomatous pattern; scalloped rows of epithelial cells rest on a layer of fusiform connective-tissue cells. Periodic acid-Schiff technique. × 200

PLATE 121

FIGURE 8.—Tumor 145, second generation. On *left* tumor is composed of fusiform cells and resembles fibrosarcoma. On *right* are glandular spaces lined by cuboidal cells with intervening fusiform cells. × 235

FIGURE 9.—Tumor 145, second generation, subline B. A small mass of hepatoma tissue in *center left*. In remaining area, epithelial and fusiform cells are blended. × 235

PLATE 122

FIGURE 10.—Tumor 147, third generation, subline B. In *lower right* a mass of osseous tissue. In *upper left* an area of fusiform cells. × 235

FIGURE 11.—Another area from same tumor. A mass of normal-appearing lymphocytes is surrounded by a rim of hepatoma cells. × 235

FIGURE 12.—Tumor 129, seventh generation. Many hepatoma cells are enlarged, rounded and detached. This tumor was grown successfully in an ascites form. × 235

FIGURE 13.—Tumor 134, fifth generation. Tumor cells resemble anaplastic carcinoma, and are infiltrating striated muscle. × 235

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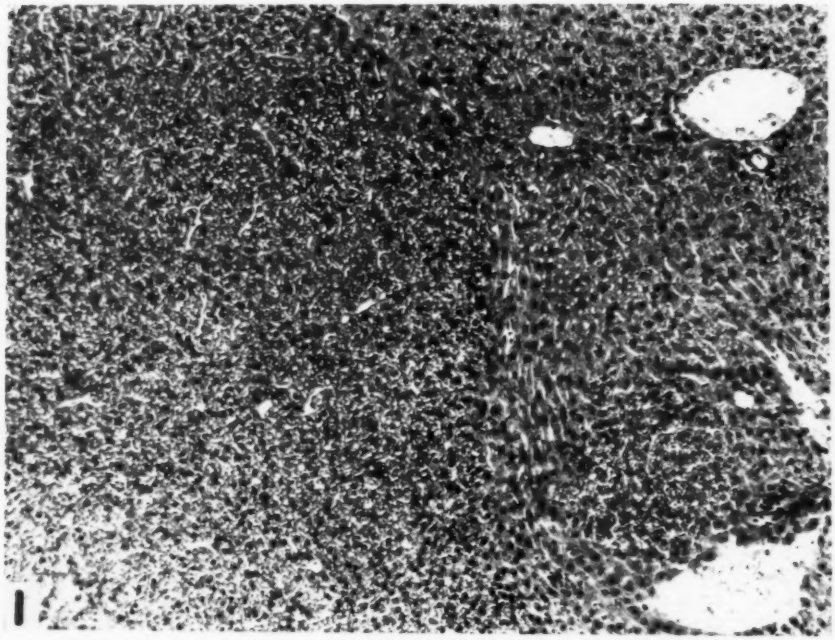
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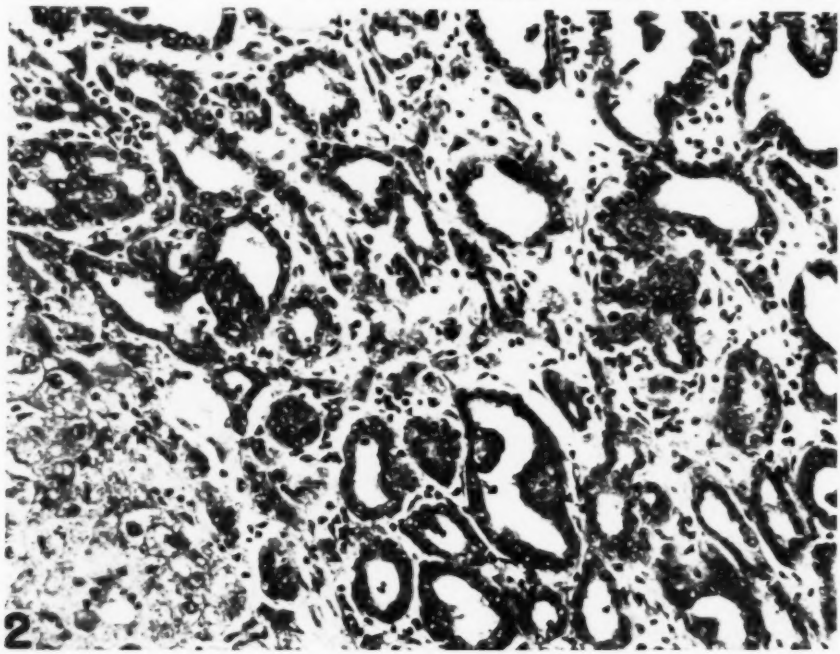
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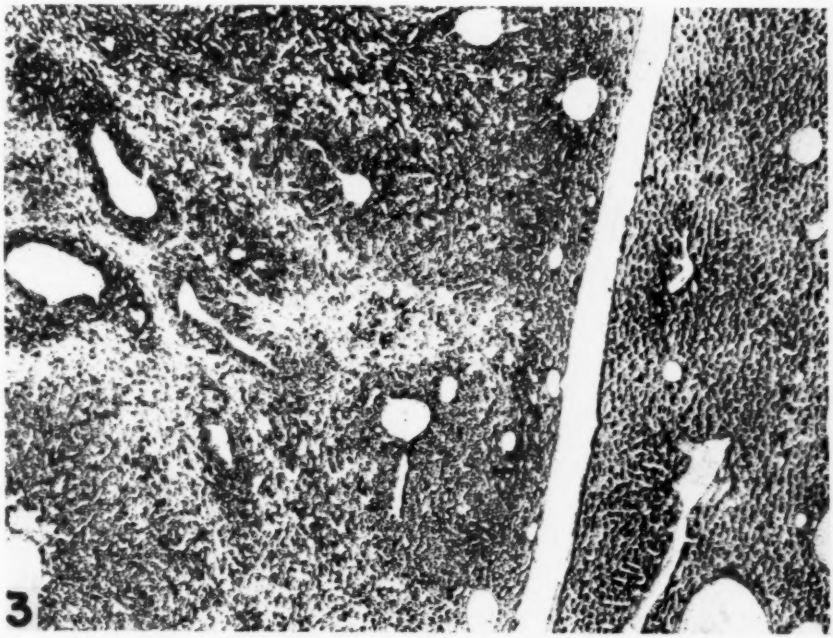

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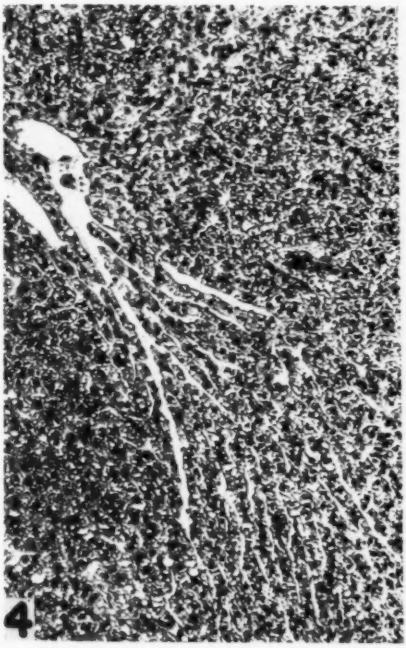

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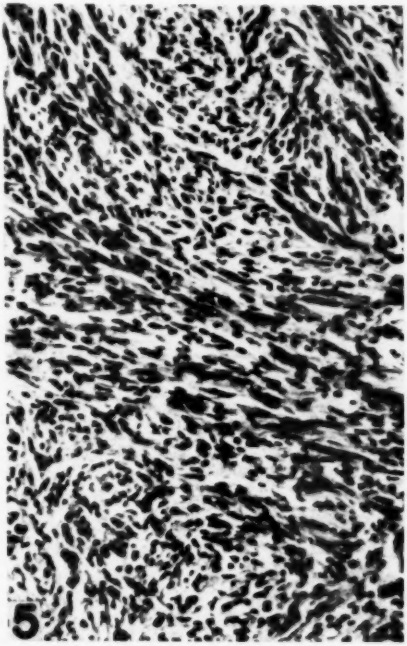

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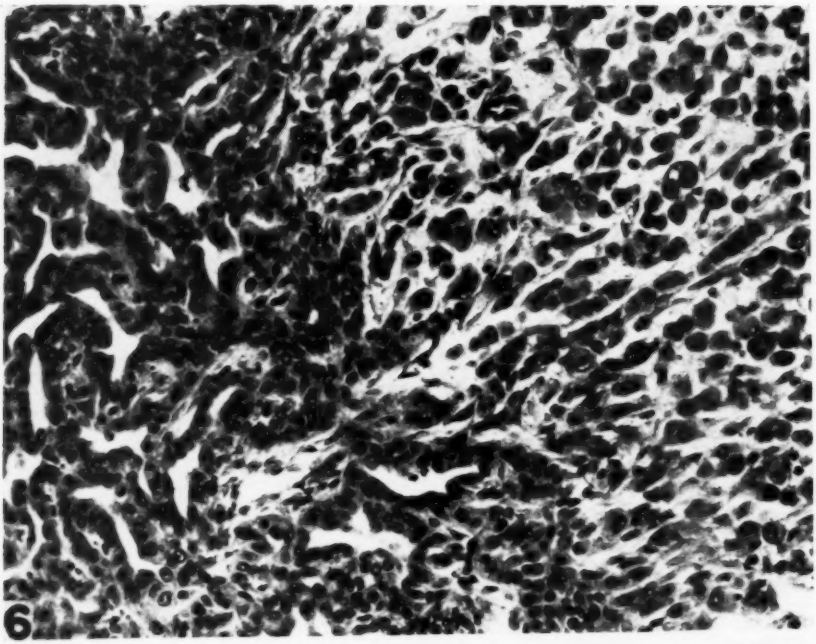
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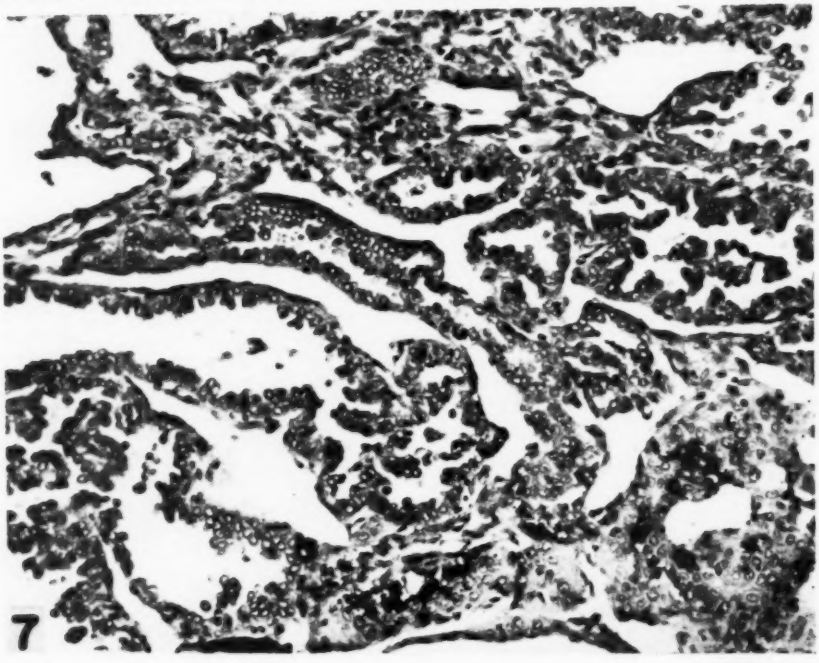
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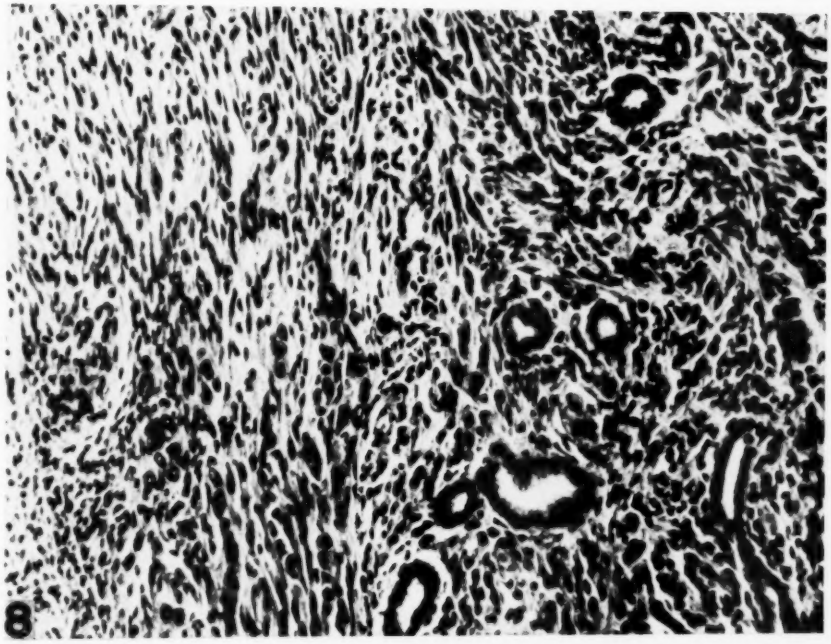

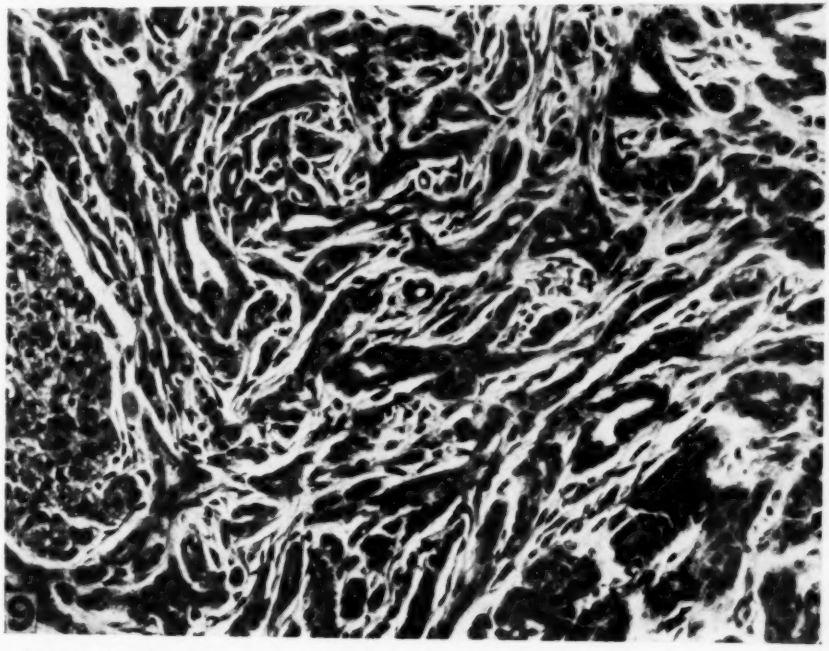

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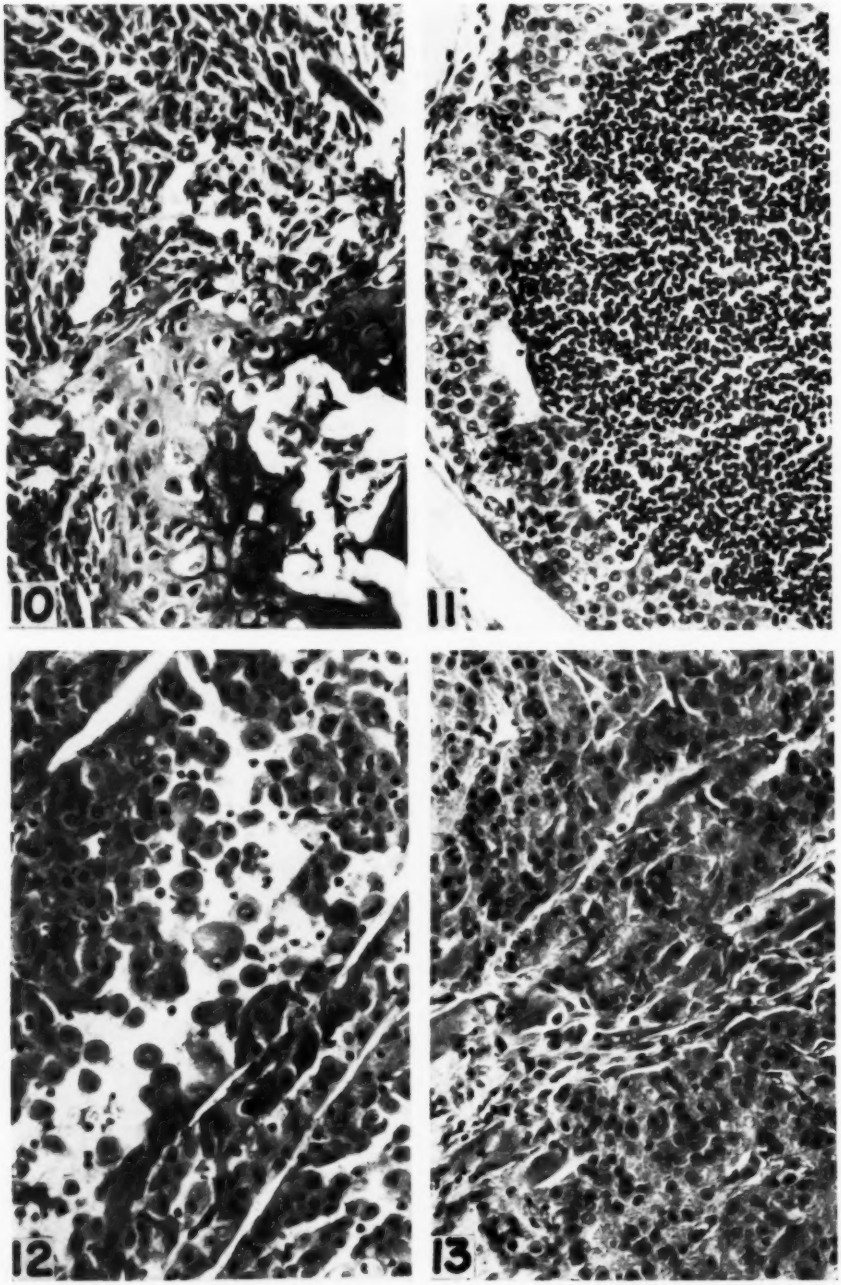
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Induction of Neoplasms by Injection of Tumor Chromatin[1,2]

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Transmission of tumors by transplanting intact viable tumor cells represents a classical method of experimental oncology. The question whether mammalian tumor cells contain an agent which, upon transfer into a new host, would transform normal cells into tumor cells, received considerable attention following the discovery of tumor-inducing viruses in certain cancers. Results with filtration of mammalian tumors were on the whole unsuccessful. There is increasing evidence that a number of enzymic activities of the cell reside in organized structures within the cell. This evidence caused us, some years ago, to raise the question as to (*a*) whether the factors responsible for the "tumor quality" of a cell might reside in one of the organized constituent structures of the cell and (*b*) whether transfer of such a cell fraction could induce, in the recipient host, transformation of normal cells into tumor cells.

Our original plan was to survey the possible activity of mitochondria, microsomes and nuclear fractions. The first experiments were carried out with tumor mitochondria; a small number of tumors were induced. Subsequently, a much higher incidence of tumor induction was obtained with chromatin, and further work was limited to this fraction. The low incidence of "takes" with tumor mitochondria and the higher incidence with chromatin suggested the possibility that the mitochondrial fraction was contaminated with varying amounts of chromatin; such contamination was regarded as probable with the techniques employed (*1*). This presentation is concerned with the transmission of tumors by tumor chromatin; some of this work has been reported previously (*1*, *2*).

Materials and Methods

The method of preparation of the chromatin fraction has been described previously (*1*, *2*). As will be discussed later, it has been found to be particularly important that all procedures be conducted at low temperatures, either in a cold room (2–4° C.) or in vessels immersed in an ice bath during all manipulations; centrifugation is carried out in a refrigerated centrifuge (International).

Two tumors have been used extensively; one is a lymphosarcoma (Murphy), obtained some years ago through the kindness of Dr. J. Murphy. This tumor has been

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maintained in our laboratories in Carworth-Wistar rats for several years. The second is a liver tumor.[4]

Results

Lymphosarcoma.—Positive results were obtained in 33 percent of rats injected with chromatin (table 1). Histologically, the tumors were indistinguishable from those transmitted by implantation of tumor-cell suspensions (*2*). In several instances, chromatin-induced tumors were used as donor material for obtaining cell suspensions or chromatin fractions. Table 1 also records the incidence of leukemia following subcutaneous implantation of either tumor cells or tumor chromatin. In no instance was a leukemic blood picture observed; the occurrence of systemic, leukemic changes in addition to the local tumor was indicated by leukemic infiltrations in liver, spleen, kidney, etc.

TABLE 1.—*Incidence of lymphosarcoma and leukemia following subcutaneous injection of lymphosarcoma chromatin and lymphosarcoma cells*

Fraction	No. of expt.	Tumor	Leukemia
Chromatin	134	44 (33%)	19 (43%*)
Cells	262	202 (77%)	25 (12%*)

*Percent figures = percentage of leukemia incidence in total takes $\left(\frac{\text{No. of leukemias}}{\text{No. of local tumors}} \times 100\right)$.

Hepatoma.—Pertinent data are presented in table 2. Tumor-cell suspensions as well as tumor chromatin preparations were injected at two sites, (*a*) subcutaneously and (*b*) intrahepatically. Whereas subcutaneous injection of tumor-cell suspensions "took" in a high percentage of cases, no tumors developed following subcutaneous injection of chromatin. However, intrahepatic injection of chromatin yielded tumors in 8 out of 29 experiments (27%), in which the temperature was kept at 2 to 4° C. during the entire process of preparation of chromatin fraction. As is indicated in table 2, the chromatin fraction was "inactive" when prepared at room temperature, which in the summer rose at times to 38° C. On the other hand, cell suspensions caused tumor growth equally well when prepared either at 2 to 4° C. or at room temperature.

TABLE 2.—*Incidence of hepatoma in A × C rats*

Fraction	Injection of fraction			
	Intrahepatic		Subcutaneous	
	No. of expt.	Hepatoma	No. of expt.	Hepatoma
Chromatin, 2–4° C	29	8 (27%)	15	0
Chromatin, room temp	36	0	14	0
Cells, room temp	22	22 (100%)	22	20 (91%)

The Question of Cell Contamination of the Chromatin Fractions

Any conclusions to be drawn from these experiments are valid only if the chromatin fractions are not contaminated with viable tumor cells. Obviously, the absence of

[4] The liver tumor used in these experiments was induced in A × C strain rats by ingestion of 2-diacetylamino fluorene. The transplantable tumor was obtained from Dr. H. P. Morris of the National Cancer Institute. It produces 100 percent "takes" when inoculated into rats either of pure A × C strain or F_1 hybrids. We express our thanks to Dr. Morris for supplying the original tumor and for continued interest and support of the research by frequent supplies of A × C and F_1 hybrid recipient animals.

cells cannot be proved unequivocally by direct methods. In table 3 are listed the the experiments which were conducted in order to obtain indirect evidence of the absence of tumor cells in the chromatin fractions.

TABLE 3.—*Experiments to test cell contamination*

1) Microscopic examination of fractions (counting chamber; serial sections).
2) Cells added to fractions; microscopic examination.
3) Leukemia incidence with cells *versus* chromatin.
4) "Inactivation" of chromatin by higher temperature.
5) Hepatoma-chromatin subcutaneous *versus* intrahepatic.
6) Anterior eye chamber inoculation.
7) Genetic experiments with mouse leukemia.

1) In the microscopic examination of chromatin fractions, as reported previously (*1*, *2*), no tumor cells were seen in smears of chromatin fractions from lymphosarcoma (80 experiments) or from hepatoma (30 experiments). In all of these experiments, portions of the packed fractions were also fixed and embedded in paraffin; serial sections failed to reveal cells. In addition, 45 grams of lymphosarcoma were used for preparation of chromatin and the entire fraction fixed, embedded in paraffin, and sectioned serially; no cells were detected.

2) Suspensions of intact lymphosarcoma cells, varying in number from 500 to 6,000,000, were injected into rats. It was found that a minimum number of 3,000 cells was necessary to induce tumor growth comparable in size and time of development to that observed in the chromatin-induced tumors. Subsequently, 3,000 tumor cells were added to a chromatin suspension, and the mixture was recentrifuged. Cells were easily recognizable in smears. It appears highly improbable that this number of cells would have escaped detection consistently.

3) As recorded in table 1, leukemia was found in 12 percent of the rats that developed local lymphosarcoma following subcutaneous inoculation of cells. The incidence was 43 percent following subcutaneous inoculation of tumor chromatin. Had the lymphosarcoma induced by the latter been caused by intact cells contaminating the chromatin fraction, a difference of leukemia incidence in this direction would not be expected.

4) Data presented in table 2 indicate that hepatoma cells exposed to room temperature remain viable, and induce tumors in a high percentage of inoculated rats. In contradistinction, chromatin inoculation caused tumor development only when the preparation was carried out at low temperatures; exposure to room temperature "inactivated" the chromatin. This again militates against the assumption that tumors induced by the chromatin fraction resulted from transfer of contaminating viable cells.

5) A further argument against cell contamination is based on the fact that hepatoma-chromatin fractions never induced tumors when injected subcutaneously, whereas hepatoma cell suspensions injected subcutaneously resulted in takes in 91 percent. On the other hand, hepatoma-chromatin fractions induced hepatoma development in 27 percent of animals inoculated intrahepatically with the fraction. Other experiments indicate that the hepatoma cells produce tumors equally readily in the subcutaneous tissue and the liver.

6) Experiments are now in progress, in collaboration with J. J. Rupp, in which the following are implanted in the anterior chamber of the eye of the rat: (*a*) rat hepatoma cell suspensions in various concentrations; (*b*) normal rat liver and hepatoma chromatin; and (*c*) hepatoma chromatin alone. In the latter animals chromatin is also injected into the liver. Final results are as yet not available. However, no tumor has developed following intra-ocular implantation of chromatin alone in animals observed for a period of 6 weeks. Tumor-cell implantation was followed by rapid tumor

growth, with the eye protruding markedly within 2 or 3 weeks. Significantly, these tumors develop following implantation of as few as approximately 500 cells. In view of the fact that no tumor developed with chromatin inoculation, contamination of this fraction with even a small number of cells appears unlikely.

7) In collaboration with J. Furth an experiment was devised, based on the "genetic test" of Law (*3*, *4*). Chromatin fractions were prepared from leukemia (lymphoma) cells of mice of the AK strain. This chromatin was injected into F_1 mice of a cross of AK × RF mice. It was planned to transplant lymphomas developed in F_1 hybrids into the two parent strains. According to Law's scheme, "takes" in the AK strain would indicate that the tumor induced in the F_1 mice originated not from cells of F_1 mice, but from AK tumor cells, transmitted with the chromatin. In our experiments we were unable to induce tumors in the F_1 mice with AK tumor chromatin. Whereas this clearly indicates the absence of cells in these chromatin fractions, the latter were inactive and consequently the experiment could not be carried through.

Independently, G. Klein carried out similar experiments using a DBA mouse lymphoma and a C3H mouse lymphoma. Klein obtained tumors following inoculation of tumor chromatin into F_1 hybrids; on microscopic examination of the chromatin fraction (smears) no tumor cells were seen. The tumors induced in F_1 hybrids were transplanted into the parent strains, and grew in the susceptible parent strain. For this reason Klein postulates the presence of tumor cells contaminating the chromatin fraction (*5*). The validity of this conclusion can be questioned in view of the fact that tumor chromatin derived from the susceptible parent, introduced into the F_1 hybrid, may transmit certain genetic qualities, in addition to the "tumor quality," to the recipient. A similar comment has been made by Hauschka (*6*).

It would then appear, from the experiments and observations listed in table 3, and briefly discussed in the preceding paragraphs, that no definitive evidence of contamination of the chromatin fractions by tumor cells has as yet come to light.

Discussion

If our observations are valid, they suggest that tumor chromatin, or a portion thereof, can enter a normal homologous cell (lymphosarcoma-chromatin-lymphocyte; hepatoma chromatin-liver cell), thereby inducing the normal cell to become cancerous. Marshak and Walker (*7*) have shown that intravenous injection of chromatin prepared from normal liver, stimulates mitosis in the liver, whereas other cell fractions are ineffective or inhibitory. The same authors found that P^{32}-labeled liver chromatin, injected intravenously, accumulates in the liver nuclei (*8*). Preliminary experiments in our laboratories (Rutman, Cantarow, Paschkis, unpublished) appear to confirm these findings; this study is being continued. Marshak and Walker suggested the possibility that a part of the nucleoprotein of a chromosome may be replaced by a similar but slightly different extracellular nucleoprotein, causing a change similar to an induced mutation. The tumor induction by chromatin reported here may be an example of such a phenomenon.

In our experiments, the tumor-inducing quality of chromatin appeared to be dependent on the intact structure of the chromatin thread; freezing and thawing, and even exposure to room temperature, rendered the chromatin inactive. We are currently planning to investigate the possibility that nucleoproteins obtained from chromatin may retain this activity. Preliminary experiments have thus far been negative.

No experiments other than those quoted above (*5*, *7*, *8*) using chromatin, have come to our knowledge. There are, however, observations of transfer of other organized cell constituents from one cell to another, which bear a general relationship to the data presented in this paper. In plants, migration of mitochondria has been studied, and related to "variegational diseases" (*9*). Lettré has reported that ascites tumor cells lose their plasma granules upon exposure to distilled water; implantation of such degranulated cells fails to give rise to tumor growth. Injection of isolated plasma granules is equally ineffective. However, in some instances injection of a

mixture of degranulated cells and of the granule fraction resulted in tumor growth (*10,11*). Whereas in these experiments isolated cell constituents are reported to enter cells, the basic concept differs significantly from that of the transfer of mitochondria from diseased to normal plant cells (*9*) or from that of invasion of normal cells by isolated chromatin (our observations).

The transformation of normal to tumor cells by introduction of tumor chromatin may bear a general biological relationship to the action of other "transforming agents," such as the transforming agent of bacteria; it may be significant in connection with our observations of the activity of the chromatin material that the transforming agent has been shown to be a desoxyribonucleate (*12*). Another, perhaps broadly related phenomenon, is that of tumor agent transformation in Amphibia (*13*). The authors interpret their work as indicating transfer of closely related nucleoproteins between tumor agent and normal cell particles.

Summary and Conclusions

Inoculation of rats with lymphosarcoma chromatin induced development of lymphosarcoma and leukemia; injection of hepatoma chromatin into the liver induced development of hepatomas. The validity of these observations is predicated upon the purity of the chromatin fraction. Whereas direct evidence of the absence of tumor cells contaminating the chromatin preparations is evidently impossible, indirect experimental evidence is presented which strongly militates against such an assumption.

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Dr. T. S. Hauschka, Institute for Cancer Research and Lankenau Hospital Research Institute, Philadelphia, Pa.:[1] Klein's experiments with two mouse ascites lymphomas (Cancer Res. 12: 589, 1952) were designed to clarify transmission of neoplasms through chromatin fractions. They provide strong genetic evidence for the otherwise undetectable presence of a few viable cells in his material which he prepared following Stasney, Paschkis and Cantarow (Cancer Res. 10: 775, 1950), but do not conclusively decide the true etiology of these challenging results.

The orthodox genetic argument is weakened by certain exceptions to the general "rule" that F_1 tissue is transplantable only into genotypes which carry the essential iso-antigens of both parental types. Furth, Boon, and Kaliss (Cancer Res. 4: 1, 1944) showed that leukemias of F_1 hybrids can be transplanted successfully into their parent strains.

The tumors which appeared in Klein's F_1 mice after massive intraperitoneal injection of chromatin from C3H or DBA lymphoma, were almost all *solid* growths, while Hauschka obtained *ascites* after inoculation of only 20 to 30 intact viable cells of the same two tumors. Does this perhaps suggest that intimate physical contact between chromatin fraction and receptive host-cell—such as would occur in the needle path through solid tissue, but not in the peritoneal fluid—is essential for the conversion of normal lymphocytes into malignant cells?

The sequence of events through which such a conversion is realized might be assumed to involve:

1) *Incorporation* of functional chromatin elements or of a self-reproducing or infectious cytoplasmic entity released during the preparatory cell grinding. Uptake of particulate material by mammalian cells of several types has been documented by Lettré, Gey, and others.

2) *Change* of at least one cell, but—judging from the short latent period—several normal F_1 lymphocytes, into neoplastic elements. This would seem to presuppose that the malignant change is inherent in a portion of the chromosomal material of the chromatin fraction or in a contaminating cytoplasmic entity. If functional chromosomal elements are involved, it must be assumed further that—after getting into the cell—these specific genetic entities are incorporated into the nucleus and equally distributed among daughter cells in subsequent mitoses.

3) Uptake of functional lymphosarcoma-chromatin fraction by normal F_1 lymphocytes, which have thereby become neoplastic, would also have to *neutralize the iso-antigenic character* of those histocompatibility factors which entered into the susceptible F_1 mouse through the nonsusceptible parent strain, the latter being 100 percent refractory to the tumor furnishing the chromatin fraction. Granting the mechanical premise of chromatin uptake, the possibility of antigenic modification by specific iso-antigenic entities in the chromatin fraction is in harmony with our demonstration (Hauschka and Levan, Exper. Cell Res. 4: 457–467, 1953) of a consistent interrelationship between aneuploidy and decreased transplantation specificity. Incorporation of antigen-carrying chromatin fraction could conceivably make an F_1 lymphocyte aneuploid with respect to the specific gene action which controls antigenic end-products and, thus, would tend to favor transplantability *beyond* the limits of genetic expectation, at least into the susceptible parental genotype. The following cytologic data may be relevant here (Levan and Hauschka, Hereditas 39: 137–148, 1953): Although nuclear fragmentation and lobation is a normal feature of the mitotic cycle of some mouse lymphosarcomas, a mechanism exists for the reconstitution of a single normal metaphase plate, after the separate micronuclei have undergone synchronous prophases. Anaphase is normal and telophase culminates again in nuclear lobation and fragmentation. Bits of chromatin material with functional kinetochores, which manage to get into an F_1 lymphocyte, could behave like the above micronuclei of lymphosarcoma 6C3HED and become part of the functional genome of the cell, rendering it malignant as well as increasing its transplantation range.

[1] Dr. Hauschka did not attend the Conference. His comments were added at the author's request.

Obviously, a number of assumptions are needed to circumvent Klein's classical genetic argument. It should be remembered, however, that histocompatibility genetics is no longer the watertight aggregate of "laws" it was even three years ago. Stimulating difficulties and the need for new interpretations were introduced into the field by the F_1-induced adaptation phenomenon (Barrett and Deringer, 1950); the antigenic conditioning of nonsusceptible hosts by cell-free enhancing substance (Kaliss and Snell, 1951); the influence of heteroploidy on the antigenic specificity of grafts (Hauschka, 1952); the *in utero* modification of mouse embryos to accept, in later adult life, persisting skin homografts to which controls are genetically refractory (Billingham, Medawar, and Brent, 1953).

It is, therefore, not unthinkable that specific chromatin fractions might render F_1 cells genetically compatible with the donor type of the chromatin.

More crucial experiments are needed to rule out the presence of viable cells in the tumor-inducing chromatin preparations. This could perhaps be accomplished through the use of cell-impenetrable "Millipore" diffusion chambers, such as have been employed by Grobstein, Algire and others at the National Cancer Institute. After such a chamber, containing tumor-chromatin fraction, has been inserted into the peritoneal cavity of a compatible animal, no tumor should arise inside the chamber if the chromatin fraction contains no cells. If, on the other hand, the fraction is contaminated with cells, then a tumor should start to grow within the chamber. Controls could include comparable tests with cell-penetrable "Millipore" diffusion chambers and deliberate contamination of chamber contents with small, known numbers of viable malignant and nonmalignant cells.

Dr. Alex B. Novikoff, Waldemar Medical Research Foundation, Port Washington, N.Y.: Klein (Cancer Res. 12: 589, 1952) injected so-called chromatin threads from tumors of inbred mice of a given strain into hybrids between that strain and a second strain. Since the tumors which arose were transplantable to mice of the first strain, Klein concluded that surviving cells were most probably present in the chromatin thread preparations because, "Tumors arising in F_1 hybrids are known not to be transplantable to any of the parent strains but only to similar F_1 hybrids." (Klein, p. 590.)

This argument rests upon the assumption that cell heredity is based exclusively on nuclear constitution. But data from a variety of fields make the universal validity of this assumption unlikely.

Two recent examples from experimental embryology, presented at a conference of the New York Academy of Sciences, October 8–9, 1954, may be cited: 1) Wilde showed the transformation of ventral ectoderm cells of post-gastrula urodele embryos into melanoblasts when a small amount of phenylalanine was added to the tissue culture medium. 2) Niu described the transformation, in cultures of urodele embryo tissue, of xanthoblasts into melanoblasts. Presumably the entry into the xanthoblast of particles from the melanoblast permanently altered the heredity of the cell. This is reminiscent of the work of Billingham and Medawar (reviewed in Biol. Rev. 22: 360, 1947); these authors considered that the conversion of white cells into black cells, in grafted guinea pig skin, was due to the transmission of cytoplasmic material from black dendritic cells into the contiguous white dendritic cells.

In these examples, the altered cells are cytologically true-breeding, and their transformation may well involve altered antigenic properties of the sort at play in tumor transplantability.

Alteration of the antigenic traits of cells by cytoplasmic and environmental factors has been described in *Paramecium* by Sonneborn and Beale (Am. Nat. 82: 69, 1948 and Genetics 37: 62, 1952). The cytoplasm, depending upon external conditions such as temperature, brings into action one of a series of non-allelomorphic genes determining antigenic type.

Among bacteria, the heredity of recipient cells may be transformed into that of donor cells by entry of bacteriophage containing genetic material (short pieces of chromatin?) of the donor cell (Zinder, Cold Spring Harbor Symp. 18: 261, 1953). For our purpose, the most interesting example of such "transductions" is the alteration of flagellar antigen in *Salmonella* (Stocker *et al.*, J. Gen. Microbiol. 9: 410, 1953).

Finally, two pertinent examples may be cited from the transplantability field itself: 1) Barrett (Cancer Res. 12: 535, 1952) found that a change of a relatively permanent kind occurred when the "transplantation history" of a tumor was altered. By transplanting a C3H tumor to F_1 hybrids (C3H $\times$ C)—a partly foreign host—for one to 22 generations, the tumor grew in and killed many more backcross hosts (C3H $\times$ C backcrossed to C): 30 percent instead of only 8 percent. The "adapted tumors" showed the altered character even after 30 generations of transplantation in the backcross hosts. 2) Kaliss (Transplantation Bull. 2: (1), 1955) has shown that progressive growth of a tumor in a foreign host, previously injected with tissue extracts, changed the tumor's reactivity or antigenicity so that it became transplantable in the foreign host.

It should be emphasized that we are not maintaining that the chromatin thread preparations of Cantarow and Paschkis contained no surviving cells. We are simply suggesting that the theoretical premise of Klein's argument need not hold in every instance.

Some Aspects of "Hepatoma NK"[1]

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I wish briefly to present, first, some descriptive material on our transplantable liver tumor, which we refer to as "Hepatoma NK," and, second, some attempts to induce tumors with microsomes isolated from this hepatoma.

1) The tumor used to establish "Hepatoma NK" consisted of a white tumor nodule in the liver and several mesenteric metastases in a Sprague-Dawley rat fed 3-dimethylaminoazobenzene. In adenocarcinomatous areas of the primary tumor, periodic acid-Schiff-positive material [like that described by Firminger and Mulay (*1*)] was present. This material became abundant in the transplantable tumor. It was both intracellular and extracellular. It gave the conventional staining reactions of mucin and the histochemical tests for acid mucopolysaccharide characteristic of epithelial mucin. In all four sublines of "Hepatoma NK" which have been carried, three by us and one by Dr. Mary Maver of the National Cancer Institute, the mucin became less abundant after 6 to 10 months and then disappeared, 5 or 6 months later.

The tumor cells of "Hepatoma NK" gave negative reactions for three enzymes studied by staining methods: alkaline phosphatase, acid phosphatase, and esterase. Of special interest in light of Greenstein's discussion (*2*) is the completely negative reaction for alkaline phosphatase given by the tumor cells; only the blood capillaries of the tumor show a positive reaction.

2) As part of a preliminary experiment done with Mr. Stanley Burns, Jr., 5 Sprague-Dawley rats were injected intraperitoneally with microsomes isolated from "Hepatoma NK." The microsomes were prepared from a 0.88 *M* sucrose homogenate, according to our published procedure for liver (*3*), except that the residues were never washed. The microsomes were suspended in distilled water (to make a 66-percent suspension) and the suspension was frozen in a deep freeze at −20° C. It was thawed on the following day, and 0.7 ml. was injected into each animal.

In each of the 5 animals a widespread intraperitoneal tumor appeared. In all respects except in "latent period," these tumors were grossly and microscopically like those of animals receiving the usual cell suspensions. The tumor from which the microsomes were prepared had some mucin-containing cells. In at least 3 of the 5 injected animals, such mucin-containing cells were present, and in 1 of these it was much more abundant than in the original tumor. Whereas it took 10 days for the animals receiving cell suspensions to become moribund from the tumor, it took 40 days for those injected with microsome fraction to become moribund.

Repeated attempts to duplicate these results have failed. In a total of 16 experiments, 50 animals were injected with microsomes prepared from sucrose homogenates and suspended in distilled water. These included filtered microsomes in 20 animals, Seitz- or Selas-filtered preparations in 16 animals, and microsomes treated with ultrasonic vibrations for 10 minutes in 14 animals. The microsomes were injected into the

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livers of 16 of the animals and intraperitoneally in the remainder. Seven of the animals injected were newborn rats, 19 hours of age.

In these 16 experiments, other preparations were tried, also unsuccessfully. Fifty-eight animals were injected with either "nuclear fraction" or "chromatin thread" preparations, and 15 with mitochondria fractions.

The tumors used in these 16 experiments were histologically like that of the first experiment in which positive results were obtained. In at least one of the later experiments, the tumor included mucin-containing cells such as were present in the tumor of the first experiment.

We feel we should continue the attempts to duplicate the initial experiment since the following experiments make the presence of any intact cells in the preparation used in this experiment unlikely: 1) The material used remained in the supernatant fluid of four successive 10-minute centrifugations at centrifugal forces increasing from 670 to 22,000 g, and one 70-minute centrifugation at 111,000 g. 2) Painstaking examination with the phase-contrast microscope failed to reveal a single intact cell in the preparation; it showed a few mitochondria and a very rare free nucleus. 3) The microsomes used were suspended in distilled water and the preparation was frozen prior to use. Survival of a viable cell under these circumstances seems quite remote from an experiment in which 0.9 percent saline, 0.25 *M* sucrose, and distilled water homogenates were injected into rats. When unfrozen homogenate was used, tumors were obtained in 9 of 16 cases with 0.9 percent saline and in 5 of 9 cases with 0.25 *M* sucrose, but in only 1 of 17 cases with distilled water. When frozen homogenate was used, no tumors were obtained regardless of whether the material was prepared with sucrose, saline, or distilled water.

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Hepatocarcinogenesis by 2-Acetylaminofluorene and Related Compounds Including Comments on Dietary and Other Influences[1]

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Two general types of chemical carcinogenic action occur—one at the site of application of the carcinogen (*1*) and one at a site remote from the portal of entry (*2*). The second type of carcinogen may be divided into a) those compounds acting on a specific organ or tissue, *e.g.*, such as the liver in rats fed 4-dimethylaminoazobenzene and certain of its analogues and derivatives and b) those compounds acting on several organs or tissues at remote sites frequently including the liver and several other organs or tissues. A good example of the latter type of carcinogen is 2-acetylaminofluorene (2-AAF) which has a high carcinogenic activity in the liver of rats and other species.

Most of my discussion will be centered on studies of 2-AAF and a number of analogues and closely related compounds which we have studied in our laboratory during the last several years. No comprehensive review of the literature of 2-AAF carcinogenesis will be attempted. My emphasis will be on the carcinogenic action of these compounds on the liver of rats, including the effects of certain derivatives which materially alter hepatocarcinogenesis, some studies of 2-AAF on other species, the metabolism of 2-AAF, and some nutritional and hormonal effects on carcinogenesis.

Hepatocarcinogenesis by 2-AAF.—(*a*) *Rats:* A summary obtained from the studies of several investigators (*1–6*) of 2-AAF-induced liver tumors shows that the number of animals developing liver tumors varies over the wide range of 0 to 100 percent (table 1). The absence of liver tumors in the Sprague-Dawley-Holtzman female and the 80-percent incidence of liver tumors in males of that strain after 8 months of ingestion of 2-AAF, Miller *et al.* (*5*), show the greatest difference between sexes. Resistance to liver-tumor induction in females of this stock may be indicated, but a longer experimental period would probably have resulted in some liver tumors in Sprague-Dawley females. Bielschowsky's (*2*, *3*) experiments indicate a much higher liver-tumor incidence in male rats in both piebald and Wistar strains. Morris *et al.* (*6*) found twice as many tumors in male Buffalo rats as in females ingesting a diet containing 0.05 percent 2-AAF. Painting resulted in a very low (11%) liver-tumor incidence in the female and none in the male. Wilson *et al.* (*1*) obtained 20 to 22 percent liver tumors with carcinogenic levels of 2-AAF in Slonaker male and female rats. They also fed 2-AAF at sufficiently low levels so that no tumors developed. Many experiments carried out in our laboratories have used only female rats because of the infrequency of mammary tumors induced in male rats by distant acting chemicals. This sex difference must be considered in the interpretation of induced liver tumors in rats.

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TABLE 1.—*Liver tumors induced by 2-AAF**

No. of rats	Strain	Sex	Dose (*percent*)	Route of admin.	Avg. length of expt. (*months*)	Avg. time AAF admin. (*months*)	Liver tumors (*percent*)	Reference
25	S	M & F	0. 125	Diet	6	5	None	Wilson *et al.*, 1941 (*1*)
5	"	M	. 062	"	5	5	20 Ca	
9	"	M & F	. 031	"	6½	6½	22 Ca	
34/41	W	M	4 Mg.D	Oral	6 to 9½	7	83 Hep†	Bielschowsky, 1944 (*2*)
11/36	"	F	"	"			83 Hep†	
5/5	"	M	"	"			100 Hep†	
5/11	"	M	"	"			45 Hep†	
13	Pi	M	4 Mg.D	Oral	6½	6	77 Hep†	Bielschowsky, 1946 (*3*)
25	Pi	F	"	"	7½	6	40 Hep†	
6	S	M	0. 016	Diet	10	10	Same as 1st expt.	Wilson *et al.*, 1947 (*4*)
6	"	M	. 008	"	13½	13½		
5	"	F	. 004	"	21½	21½	1 ? Nod.	
5	"	M	. 001	"	24½	24½	None	
9	H	F	0. 036	Diet	8	8	None	Miller *et al.*, 1949 (*5*)
10	H	M	. 036	"	8	8	80	
9	M(7) W(2)	M & F	0.12 mg.D	P	16½	16½	11 Ch	Morris *et al.*, 1950 (*6*)
10	B	M	. 05	Diet	7½	5½	60 Hep	
10	B	F	. 05	"	7½	5½	30 Hep	

*B=Buffalo; Ch=Cholangioma; D=daily; H=Holtzman; P=skin painting; Pi=piebald; M=Minnesota; S=Sionaker; W=Wistar.
†Benign cholangiomas present in nearly all animals.

(*b*) *Mice:* Armstrong and Bonser (*7*) studied 2-AAF tumor induction in male and female mice of five strains: CBA, IF, RIII, White label, and Strong A. The percentage of benign and malignant hepatomas was 73, 44, 16, 10, and 5, respectively. Most of the tumors arose between 1 and 1½ years. The dosage was 6 mg. per mouse per week by stomach tube. Malignant hepatomas were noted only in 3 CBA males and 2 males and 1 female of the IF strain. Mice of C3H, C57BL, and A strains ingesting 2-AAF in our laboratory appeared more resistant to hepatoma induction than rats. Tumors arose after a longer feeding period but transplantable liver tumors were produced in all 3 strains, Morris (*8*).

(*c*) *Dogs:* Dogs of either sex are susceptible to the development of hepatomas after the ingestion of 2-AAF. Our induction period ranged from 68 to 91 months (*9*). Allison *et al.* (*10*) report development of tumors in the liver of dogs after a 2-AAF feeding period of only 6 months, but the lesions observed may not have been hepatomas.

The amount of 2-AAF ingested by our dogs was 8.3 to 10.5 mg. daily or 5 to 6 times the amount (1.6 mg. per kg. body weight) ingested by rats for a period of 18 weeks—an amount just sufficient to develop approximately 5-percent tumors. A large amount of orally administered 2-AAF is necessary to induce tumors in dogs, and there is a long latent period. Weisburger *et al.* (*11*) using isotope dilution techniques have isolated 7-OH-2-AAF and 2-AF from dog urine. Both metabolites were present in the urine to the extent of 1 or 2 percent of the amount ingested in a single dose. Twelve percent of 2-AAF was present and 12 percent of 7-OH-2-AAF was conjugated with glucuronic acid. Their experiments suggest that the dog may not only deacetylate 2-AAF but may also oxidize 2-AAF by the same pathway as does the rat, as shown by Bielschowsky (*12*).

Hepatocarcinogenesis by 2-diacetylaminofluorene (2-diAAF).—One of the most rapidly acting ingested carcinogens in our hands has been 2-diAAF. Morris *et al.* (*6*) obtained 100-percent hepatomas in 12 rats (7 female and 5 male) of the Minnesota strain after ingestion of 0.05 percent 2-diAAF. The average survival was 58 days. All the males and 2 females were autopsied in the fifth week, and 3 females in the ninth week. The remaining 2 females survived 21 weeks. Painting with 2-diAAF was much less effective in inducing liver tumors.

2-diAAF fed to A × C strain male rats for 2 periods of approximately 4 weeks each, with a rest period of 3 weeks between the carcinogen feeding periods (*13*), produced hepatomas following the first carcinogenic feeding period, or beginning at approximately 6 weeks from the start of the experiment. The incidence of hepatomas in male rats increased rapidly to a maximum of 75 percent after 12 weeks. Earlier lesions appeared and regressed on withdrawal of the drug. The A × C female rats subjected to the same treatment were almost completely free of hepatic lesions after 22 weeks. Castrated females given testosterone approached normal males in incidence of liver tumors, while castrated males were intermediate but were more nearly like normal females.

The frequency of administration of 2-diAAF in a second experiment influenced the time and incidence of liver tumors. The drug-containing diet was alternated every other week with the drug-free diet. The final incidence of liver tumors in castrated female rats given testosterone on this regimen was not significantly different from the first experiment, but the liver tumors appeared much later. The diet No. 143 [Morris *et al.* (*6*)] used in these experiments contained 12-percent protein (vitamin-free casein), 74-percent dextrose, and only 0.25-percent fat, as coconut oil.

However, 15 months of feeding of 2-diAAF in a diet of natural food stuffs, well fortified with protein, vitamins and fat, was required to produce liver tumors in A × C males.

Changes in position 7.—The hydroxylation of 2-AAF in position 7 to form 7-OH-2-AAF was first reported by Bielschowsky (*12*). He believed this derivative of 2-AAF to be noncarcinogenic, the one excreted in largest amounts after 2-AAF feeding, but not the only metabolite formed. The carcinogenic properties of this metabolite were later studied by Hoch-Ligeti (*14*). She found a reduction in the incidence as

well as a delay in the time of appearance but the distribution of tumors was about the same as for 2-AAF. Survival of the 7-OH-2-AAF-fed animals was approximately 25 months at termination of the experiment so that some tumors observed could have been of spontaneous origin.

Halogen substitution in position 7.—The halogen fluorine incorporated into the 7-position by Miller *et al.* (*15*) greatly increased hepatic carcinogenic activity in both male and female rats.

The 7-chloro-2-AAF, on the other hand, has some carcinogenic action on the liver in female Buffalo strain rats, Morris (*8*). Our animals survived only 2½ months because the chemical was quite toxic. Additional tests are indicated. The 7-iodo-2-AAF, however, is without carcinogenic effect at any site. The substitution of a halogen in the 7-position would be expected to interfere with oxidation at that position.

Position 3.—The 3-iodo-2-AAF produced by substitution of a halogen in the 3-position was noncarcinogenic to the liver, but was much more specific for the acoustical duct, inducing a large number of squamous-cell carcinomas.

3-AAF ingested in another, as yet incomplete experiment, has produced a hepatoma, a kidney adenoma, and an adenocarcinoma of the uterus all in one rat. Five other animals were negative. Dr. Elizabeth Weisburger (*16*) isolated two metabolites, 3-AF and 2-OH-3-AAF, in the urine of rats ingesting 3-AAF. These results indicate that deacetylation and *ortho*-oxidation take place in the rat ingesting 3-AAF. We do not yet know whether hydroxylation occurs on the ring not containing the acetylamino group or on the 4-position of the ring. If the former takes place, 7-OH-3-AAF would likely be formed.

Positions 1 and 4.—The 4-AAF was synthesized by Weisburger *et al.* (*17*) and has been tested for carcinogenicity. It seems to be entirely without carcinogenic activity when administered orally to the rat. The 1-AAF has produced no tumors of the liver, mammary glands or ear duct, but we have found in autopsies of 17 females of the Buffalo strain, 1 adrenal, 2 pituitary, 1 uterine, and 1 ovarian tumor after 7 to 20 months.

Alterations on the acetyl group.—*N*-2-fluorenylsuccinamic acid induced 5 hepatomas in 6 female Buffalo rats in 17 autopsies. No other types of tumors were observed. The results with these last two compounds, 1-AAF and *N*-2-fluorenylsuccinamic acid, do not support the observations of Miller *et al.* (*15*) that compounds failing to produce mammary tumors in female rats would also fail to produce tumors at other sites.

Halogenation on the ω-C of the acetyl group.—Replacing all three hydrogens by fluorine on the ω-C of the acetyl group of 2-AAF produces a most remarkable carcinogenic effect. This trifluoro-2-AAF derivative was not only much more carcinogenic to the liver, but also produced many tumors of the mammary gland and the acoustical duct in female Buffalo rats. No increase in carcinogenic activity but rather a retarding carcinogenic action appears to occur in an incompleted experiment in which one hydrogen only on the ω-carbon was replaced by fluorine.

Effects of N-methylation.—The carcinogenic action of 4-dimethylaminoazobenzene (DAB) appears to be dependent on at least one *N*-methyl group, as noted by Miller and Miller (*18*), but in our laboratory 2-dimethylaminofluorene fed to female Buffalo rats produced 10 hepatomas, and monomethylaminofluorene produced 8 hepatomas and 1 cholangioma each out of 17 autopsies—essentially no difference between the two. These results are in contrast to those of Bielschowsky and Bielschowsky (*19*), who observed no liver tumors in female piebald or Wistar strain rats but found more hepatomas in males of both strains after ingesting the monomethyl-AF than the diemthyl compound. The 2-monomethyl-AF in our studies was fed for an average of 10½ months (range 7–17½ months). The 2-dimethyl-AF was fed for an average of 11 months ranging from 8 to 13 months. The animals were kept on the basal diet for an average of 3 more months after cessation of drug-feeding. Bielschowsky's experiments went for 12 to 15 months.

Studies were carried out by Weisburger *et al.* (*20*) to obtain additional information on the metabolism of 2-methyl-C^{14}-aminofluorene. Fifty-five percent of the activity in

the respiratory CO_2, 19 percent in the urine, and 12 percent in the feces appeared 72 hours after a single dose. Most of this activity was eliminated during the first 24 hours. The isolation of radioactive choline and serine from the liver and carcass, and creatinine from the urine, proved the occurrence of demethylation. It was further shown that most of the urinary activity was present in preferentially water-soluble form, while only a trace of unchanged compound was excreted. The demethylation of 2-methylaminofluorene will form the metabolite AF. It must be presumed, therefore, that AF or some AF metabolite is the active carcinogen.

Structural alteration at the 9-position.—Miller *et al.* (*5*) replaced the CH_2 bridge of 2-AAF by –S–, S or –O–. Eight months of feeding to Sprague-Dawley-Holtzman

stock rats produced no tumors of the liver in either male or female animals. Morris, Velat and Wagner (*21*), however, found in autopsies of 17 female Buffalo-strain rats ingesting 2-AA-fluorenone, 1 hepatoma after 20 months and one lymphosarcoma of the liver after 19 months. Eliminating the azo linkage of DAB and uniting the two benzene rings with a single bond gives 4-dimethylaminobiphenyl, a compound more related to 2-AAF than to the 4-dimethylaminoazobenzene. Miller *et al.* (*5*) fed 4-dimethylaminobiphenyl for 10 months producing only 3 benign hepatomas in 10 rats, although –S–, S or –O– containing compounds produced tumors of the mammary glands and acous-

tical duct. 2-AAF used as a control produced in 8 months a high incidence of liver tumors in male but none in female rats (see table 1).

Walpole *et al.* (*22*) made subcutaneous injections of the demethylated derivative, the 4-aminobiphenyl, in arachis oil for 8 to 12 months. Three liver lesions developed in 23 rats—one a hepatoma, one a benign cholangiomatous cyst, and one a metastasizing sarcoma (primary to liver).

Demethylating the 4-dimethylaminobiphenyl to give 4-aminobiphenyl does not materially change the carcinogenic properties to the liver but increases the incidence of intestinal tumors, Walpole *et al.* (*22*). 4-Aminobiphenyl when acetylated gives 4-acetylaminobiphenyl, a compound no longer carcinogenic to the liver (*21*) but one which on ingestion produces a high incidence of mammary tumors.

The 4-acetylamino-4′-fluorobiphenyl, on the other hand, is an active hepatocarcinogen in female Buffalo strain rats inducing 11 hepatomas in 16 autopsies (*21*). This compound has been exceedingly active in producing mammary cancer. Tumors of several other sites were also noted after a 12-month feeding period.

As noted for the aminobiphenyl compounds, except for the 4-acetylamino-4′-fluorobiphenyl, a single linkage between the two benzene rings may drastically decrease their carcinogenic effect on the liver of rats. If we interpose an ethylenic linkage between the two benzene rings as occurs in 4-dimethylaminostilbene, a more toxic compound results, Haddow *et al.* (*23*). The 4-dimethylaminostilbene is not a specific carcinogen for the liver because it produces other types of distant tumors including mammary and acoustical-duct tumors.

Elson (*24*) found 4-dimethylaminostilbene given to rats on a 5-percent casein diet supplemented with 0.4-percent methionine favored the development of cholangiomas in male rats (7 in 13 rats examined post mortem) but not other types of tumors. The liver tumors also appeared earlier (3–11 months) than other types of tumors. The chemical in arachis oil was administered by intraperitoneal injection. 4-Dimethylaminostilbene and 2′-chloro-4-dimethylaminostilbene both, after intraperitoneal injection, induced a variety of distant tumors; 15 different organs were involved but no sarcomas were found. Haddow *et al.* (*23*) described a large number of sarcomas after subcutaneous injection of several aminostilbenes. A 5-percent casein diet or a 20-percent protein diet derived from 11-percent casein, 72-percent wheat flour and 8-percent whole milk powder had no effect on the incidence of liver tumors or tumors of other sites in rats given the two aminostilbenes (*24*). The route of administration,

however, of aminostilbenes affected the types and to some extent the location of the induced tumors.

Survival on different diets.—Miller *et al.* (*5*) reported improved survival of rats fed 2-AAF in a specific type of grain diet when compared to survival on a semisynthetic diet. They also noted earlier development of tumors in the animals on the grain diet.[3]

A diet of natural foodstuffs containing 2-AAF also favored the survival of rats when compared to a 12-percent casein, low fat, semisynthetic diet, Morris *et al.* (*25*). The diets of natural foodstuffs, therefore, usually resulted in a higher incidence of tumors, for it has been our experience that more tumors and a greater variety of tumors develop in rats that survive 8 to 10 or more months. Experiments carried beyond 20 months become much more complicated to interpret because of the possible development of spontaneous tumors.

Morris, Stewart and co-workers (*25*) noted similar wide variations in tumor incidence in several experiments using Sprague-Dawley male and female and Buffalo female rats. Each of their experiments was started at approximately the same time of the year. More liver tumors arose in Sprague-Dawley females when they were started on the chemical-containing diet at 1 month of age than when they were started at 4 months of age. Some suggestion of the same relationship exists for Buffalo females, but no explanation is apparent why only two mammary and no ear-duct tumors developed in one Buffalo-strain experiment. According to Symeonidis (*26*), both Buffalo and Sprague-Dawley female rats after ingesting 2-AAF have a low incidence of liver tumors but a high incidence of mammary tumors. He also noted that most of the rats free of mammary tumors developed liver tumors.

Variation in protein and riboflavine level.—Both male and female Buffalo rats were fed low fat diets containing 12-, 18- or 24-percent casein. More hepatomas developed on the 12-percent protein diet than on the 18- or 24-percent levels. The addition of "injectable-liver-extract," a source of vitamin B-12, to the 12-percent casein diet resulted in few liver tumors, possibly due to the poor survival. A high incidence of cirrhosis was noted in all these experiments with almost all rats being involved. Nodule formation occurred to some extent in most of the animals. These experiments did not prove decisive so far as effect of different levels of casein on 2-AAF carcinogenesis is concerned. The effect of casein level on development of tumors at other sites showed more mammary and ear-duct tumors on the diet containing 18-percent protein. A high incidence of ear-duct tumors developed in the 24-percent casein diet experiment, but here also no very clear-cut results on carcinogenesis were obtained.

Riboflavine at levels of 1, 2, and 10 μg. per gram of the 24-percent vitamin-free casein diet had no apparent influence on hepatocarcinogenesis in female Buffalo strain rats given 2-AAF by stomach tube (*21*).

Metabolism.—Investigations designed to shed light on responses of the organism to the intake of 2-AAF were early recognized as being essential steps in understanding the mechanism of 2-AAF carcinogenesis. The development of a quantitative chemical assay for 2-AF and related compounds by Westfall (*27*) constituted the first definite accomplishment toward that goal. Utilizing this quantitative chemical method, Morris and Westfall (*28*) observed a wide distribution of substantial amounts of diazotizable material in liver, kidney, plasma, and muscle after oral administration of 2-AAF, with the highest concentrations occurring approximately 3 hours after feeding. They interpreted their findings as a logical explanation for tumor production at sites distant from the portal of entry.

Subsequently, Morris and Westfall (*29*) found that rats excrete in their urine daily about ⅓ of the ingested 2-AAF as estimated by the diazotizable fraction. None could be detected in the urine 9 days after the last dose. Since Morris and Westfall (*29*) could account for only about ⅓ of the administered dose, the possibilities exist that 1) an appreciable amount of the ingested 2-AAF may have been transformed into

[3] Ground yellow corn, 68; linseed-oil meal, 16; powdered skim milk, 12; alfalfa-leaf meal, 2; cod-liver oil, 1; iodized NaCl, 0.5; and $Ca_3(PO_4)_2$, 0.5.

metabolites which no longer possessed the free aromatic amino group, 2) the aromatic amino group may have been firmly bound in complex linkages, and 3) the color intensity of some metabolites present may have been different from that produced by 2-AF.

Because of the inability to account for the largest portion of ingested 2-AAF by the chemical method, tracer studies were initiated by Morris, Weisburger and Weisburger (*30*) on the distribution of 2-AAF-9-C^{14} in the rat. Ninety-five percent of the administered activity was accounted for by the tracer technique. The widespread distribution of radioactivity as an indicator of the carcinogen, or its metabolic products, furnished additional support for the view that distribution of the chemical and tumor induction were in some way related.

One *in vivo* reaction of 2-AAF which has been demonstrated by Weisburger *et al.* (*31*) is the hydrolytic removal of the acetyl group after the oral administration to rats. About 40 percent of radioactivity was expired in the CO_2 during the 96 hours following a single dose of 2-AAF-ω-C^{14}. The experiments permitted no quantitative conclusions as to the extent of deacetylation other than to give a minimum value. The breath contained insignificant amounts of radioactivity 96 hours after the administration of 2-AAF-9-C^{14}. The radioactivity in the urine accounted for 65 percent of the dose of 2-AAF-9-C^{14} administered and the feces, 30 percent. The disclosure of such large amounts in the feces by this tracer technique was in sharp contrast to the failure of chemical methods used previously by Morris and Westfall (*29*) in their study of the excretion of 2-AAF, and furnished the first evidence that the feces contained substantial amounts of nondiazotizable material. Dyer *et al.* (*32*) found no evidence that the nonacetone-soluble, nondiazotizable material combined with proteins or saponifiable lipids of the tissues in combinations that were reversible upon hydrolysis. They expressed the opinion that the nitrogen from the administered 2-AAF was present in the tissues in a nondiazotizable form or in extremely water-soluble material. Further investigations of the fate of the nitrogen of 2-AAF in the organism depended on tracer studies with 2-AAF enriched with N^{15}. Dyer, Damron and Morris (*33*) accounted for an average of 65 percent of the N^{15} from 2-AAF-N^{15} in the urine and 35 percent in the feces following oral administration of the labeled compound. The recoveries of N^{15} from urine and feces were similar to those for C^{14} of 2-AAF-9-C^{14}, Weisburger, Weisburger and Morris (*34*). This finding was a significant observation because it gave substantial support to the view that the N_2 was not removed and the ring structure of 2-AF was not broken up during its passage through the organism, thus giving more assurance that by following the C^{14} activity we were in fact following a reasonably intact nitrogen-containing ring system.

The next step in our metabolism studies was to trace the pathway of the 2-AAF-9-C^{14} in the rat to obtain information about the organs mainly concerned with its metabolism. These experiments by Weisburger, Weisburger and Morris (*34*) revealed that the radioactivity appeared in the bile very shortly after stomach intubation with the labeled carcinogen and reached a constant level after the first hour, amounting to about 0.8 percent of the dose per hour during the first 30 hours. This indicated that the liver functioned at a maximum rate very soon after the compound reached it from the gastrointestinal tract. The presence of radioactivity from 2-AAF-9-C^{14} in the stomach and its absence in the feces at necropsy led us to surmise that the rate of absorption from the gastrointestinal tract limited the rate of metabolism.

The absence of activity in the feces of rats with biliary fistulas supported the conclusion that activity normally present there originated from the bile. The lowered urinary activity in rats with biliary fistulas must have been due to the removal with the bile of some metabolites usually found in the urine. The total normal urinary activity must have come from 1) removal of activity in the circulatory system (coming directly either from the liver or gastrointestinal tract) or 2) activity removed from the blood by the liver, then discharged into the bile, returned to the intestines where a portion was reabsorbed, recirculated, and finally excreted by the kidneys.

Could there be an alternative pathway for the circulation of 2-AAF? The results obtained by Weisburger *et al.* (*34*) from a rat with a lymphatic fistula followed for 114

hours indicated that only about 2 percent of the radioactivity administered was present in the lymph during a period of time sufficiently long to permit excretion of most of the radioactivity given. The liver and kidney, as indicated from these studies, were the most important organs involved in the metabolism of 2-AAF. Did any of the particulate fractions of these organs have any special affinity for C^{14} from 2-AAF or its metabolites? If they did, some clues might be obtained as to the nature of the reactions responsible for the metabolic transformation undergone by 2-AAF. No pronounced concentration of activity was found in any one particulate fraction-nuclei, mitochrondia, microsomes, supernatant or washings; therefore, no immediate conclusions can be drawn regarding the intracellular site or cellular component responsible for the metabolism of 2-AAF.

The retention of activity by the particulate fractions led to investigations to see whether proteins combined with 2-AAF in a manner similar to the findings reported by the Millers (*35*) for the azo-dye type of carcinogen. Dyer (*33*) first found liver protein-binding with the 2-AAF-N^{15}-labeled carcinogen. Subsequently the Weisburgers (*34*) noted about 50 percent of the total liver activity after 2 doses of 2-AAF-9-C^{14} with the animals sacrificed at 24 and 162 hours combined with the acetate-insoluble proteins, while about 15 percent of the total liver activity was in the soluble proteins. The specific activity of the protein decreased rather slowly in one week when no 2-AAF was administered. Hydrolysis of the radioactive protein with alcoholic KOH in an inert atmosphere and subsequent extraction with ether resulted in over 80 percent of the activity remaining in the aqueous layer and the rest transferred to the organic phase. Considerable transformation must have taken place.

Studies of the effect of riboflavine on the metabolism of 2-AAF-9-C^{14} in rats showed no significant difference with regard to protein-bound radioactivity between the groups fed the low, normal, or high level of riboflavine. The radioactivity of the upper gastrointestinal tract was significantly higher in depleted animals than in the riboflavine-supplemented or control animals, which suggests that riboflavine deficiency has a pronounced delaying action on the absorption of radioactive material. The deficient animals excreted a larger proportion of water-soluble metabolites such as glucuronides of the hydroxylated compounds and a smaller amount of the ether-soluble compounds such as 2-AF, and 2-AAF, and monohydroxylated derivatives thereof.

Some 12 metabolites of 2-AAF-9-C^{14} separated by radio chromatographic techniques by Weisburger *et al.* (*36*) have been identified as 2-AAF, 2-AF, 7-OH-2-AAF, 7-OH-2-AF, 3-OH-2–AAF, 1-OH-2-AAF and the glucuronides of these hydroxy derivatives. The relation of 2-AAF metabolism to liver carcinogenesis is not understood as yet, but because the liver is a major organ involved in the degradation of carcinogenic chemicals, we believe intensive research into metabolic phases will yield significant information on the mechanism or mechanisms of hepatocarcinogenesis.

Mechanism.—The solubility and diffusibility of the respective classes of remotely acting carcinogens has been suggested as one explanation for the difference between locally acting and remotely acting compounds. Thus a carcinogen that may be relatively inactive toward the cells at the site of administration may be sufficiently diffusible to reach distant, more responsive tissues, making it function as a remotely acting compound.

Does a remotely acting carcinogen like 2-AAF act more effectively when administered to the target organ for which it is carcinogenic at a distance? This area has been only briefly explored by Wilson *et al.* (*37*), who placed 100 to 150 mg. of crystals of 2-AF in a subcutaneous pouch in rats and an additional 250 mg. 96 days later. Initial rapid absorption and excretion were indicated by a red discoloration of the shavings that was noted for a few days following the implantations but which soon disappeared. Crystalline material was observed at the site of implantation 22 days after the second implantation but no gross lesions were noted. Small liver nodules and cysts were observed in one rat 477 days after the initial implantation but other organs were normal. Berenblum and Haren (*38*) (cited from Berenblum 1954) studied the carcinogenic activity of 2-AAF in acetone applied to the external auditory duct. No

local tumors developed. Wilson *et al.* (*39*) applied 2-AAF powder to the external ears of rats weekly for 13 months. No tumors of the ears or other sites had developed 8 to 11 months after stopping the application of the powdered 2-AAF.

Morris *et al.* (*6*) painted acetone solutions of 2-NF, 2-AF, 2-AAF, and 2-diAAF on the intrascapular region of rats. They obtained distant tumors from all four compounds, but only 2-AF induced a few tumors at the site of application. The mechanism determining where a given carcinogen will act, therefore, seems more complicated than that based on solubility or diffusibility, although a certain amount of solubility apparently must occur because 2,2′(diacetylamino)9,9′-bifluoryl has been found to be noncarcinogenic (*40*) and no evidence either of absorption or excretion in the urine of this compound has been found by Dyer and Morris (*41*). The amount administered was recovered in the feces after oral administration.

2-AAF is only slightly soluble in water, Westfall (*27*), but it appears to be more soluble in tissue homogenates (*42*). This may be explained by the observations of Neish (*43*) that purines have a distinct solubilizing action on some aromatic compounds.

The question arises as to whether the carcinogen *per se* is the active compound or some metabolite produced by the organism during the processes of metabolism of the compound administered. At the present time too little is known about the carcinogenic potency of metabolites of 2-AAF. That is one of the problems currently under investigation in our laboratory.

Summary

Liver tumors have been induced in several animal species by ingestion of 2-acetylaminofluorene (2-AAF). The liver of the male rat is more susceptible than that of the female to tumor induction by this chemical. The 2-diacetylaminofluorene (2-diAAF) derivative acts more rapidly on the liver of the male rat than 2-AAF, and this carcinogenic action of 2-diAAF to the rat liver is enhanced by testosterone and depressed by estrogen. The halogen, fluorine, incorporated in the 7-position of 2-AAF or the 4′-position of 4-acetylaminobiphenyl enhances carcinogenesis, not only of the liver but of other sites. All hydrogens in the ω-carbon of the acetyl group replaced by fluorine also enhances carcinogenicity, but the incorporation of the acetylamino group in the 1, 3, or 4 positions of fluorene decreases carcinogenicity in the rat. 2-Monomethylaminofluorene is demethylated and its hepatocarcinogenic activity must therefore be presumed to be due to AF or some active AF metabolite. Variations in dietary levels of protein gave indecisive results on hepatocarcinogenic activity of 2-AAF.

Metabolism experiments with a variety of analytical methods have shown 2-AAF to have a wide distribution in the organs and tissues of the rat. The acetyl group is rapidly removed *in vivo*, but the nitrogen of 2-AF appears to stay with the fluorene ring structure during metabolism. Radioisotope tracer studies indicate the carcinogen is absorbed from the intestine, excreted in the bile, reabsorbed and substantial amounts excreted in both urine and feces, but lymph is not an important pathway of transportation of the chemical in the rat. At least twelve metabolites including hydroxylated derivatives and their glucuronides have been separated in rat urine. Small amounts of the isotopically labeled carcinogen are bound to acetate soluble and insoluble proteins. Crystalline or powdered 2-AAF has slight, if any, carcinogenic activity, but absorption by painting an acetone solution on the skin produced distant tumors although local tumors seldom developed, indicating that the chemical must be absorbed to be carcinogenic.

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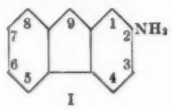
Dr. Francis E. Ray, Cancer Research Laboratory, University of Florida, Gainesville, Fla.: It is desirable at this time to emphasize several important relationships in this interesting paper. Compounds that have been shown to be converted *in vivo* to aminofluorene, I, give a high incidence of hepatomas. These are acetylaminofluorene



I

and the mono– and dimethylaminofluorenes. The succinoyl derivative is probably in the same class. Those derivatives that are difficult to hydrolyze give few or no hepatomas. The toluenesulfonyl, benzoyl, and thiazole derivatives are good examples. Modification of the solubility with consequent transportation to a particular site before hydrolysis has occurred is another possibility of directing the attack to a specific organ. Trifluoroacetylaminofluorene and diacetylaminofluorene are examples of such modification of solubility.

Nuclear substituents may change the basicity so that even the acetyl group becomes difficult to remove; for example, the 7-chloro and the 7-iodo derivaties. The opposite effect is also possible as shown by the 4-acetylaminobiphenyl which gives no hepatomas, but when inorganic fluorine is inserted in the 4′ (the *para-para*) position, the compound is also possible as shown by the 4-acetylaminobiphenyl which gives no hepatomas, but when inorganic fluorine is inserted in the 4′ (the *para-para*) position, the compound produces a large number of hepatomas as well as many other cancers. The same result has been shown by the Millers to occur in the aminofluorene series. Here, ease of hydrolysis *plus* the change in basicity leads to an enhancement of activity beyond that of aminofluorene itself.

If the amino group is moved from the 2-position of fluorene to some other (1, 3, or 4) position few tumors of the liver result. I would suggest here that the spatial configuration may be a factor but in the absence of basicity data this is only a guess.

The conversion of the methylene group (CH_2) in the 9-position to the keto group (C:O) destroys, or at least weakens, the power to cause liver tumors. It also converts the compound from colorless to red which indicates hyperconjugation. Since the replacement of 2 hydrogen atoms by oxygen largely destroys the liver-carcinogenicity of 2-aminofluorene, it is not surprising to find in the work of the Millers that the replacement of the entire methylene group by S, SO or O also destroyed this property. In all cases, however, the compounds retained the power to produce tumors at other sites in the body.

The most important conclusion that can be drawn from this important work, it seems to me, is that it brings within the field of probability the synthesis of carcinogens that will attack primarily one organ. We have seen here that 2-acetylamino-3-iodo-fluorene gave mostly ear-duct tumors; 4-acetylaminobiphenyl gave mammary tumors; 4′-fluoro-4-acetylaminobiphenyl gave many kidney tumors; and 2-acetylaminofluorenone, mammary, and uterine tumors.

Dr. Helen M. Dyer, National Cancer Institute, Bethesda, Md.: The patterns of excretion after a single dose of 2-aminofluorene (2-AF), some of its derivatives, and several structurally related substances, have been followed in the rat by the estimation of total diazotizable material (diazo test) and the diazotizable metabolites that react like 7-OH-2-AAF (nitrite test), to see if any relation exists between the excretory behavior of the compounds and their ability to induce tumors. The nitrite test detects some of the phenolic derivatives which have been found to derive from the detoxication of aromatic amines by oxidation. Some correlation does appear to exist between the degree of hepatocarcinogenicity and the amount of diazotizable R-salt-coupling material excreted in the urine (indication of absorbability), and the production of

"nitrite" detectable metabolites which behave like 7-OH-2-AAF of most of the following 2-substituent derivatives of AF:2-AF, 2-AAF, 2-diAAF, 2-NF, 2-monomethyl-AF, 2-dimethyl-AF, 7-OH-2-AAF, 2,7-diAAF, 2-benzoyl-AF, 2,2′-diacetylamino-9,9′-bifluoryl, and 2,2′-diacetylamino-9,9′-bifluorylidene. Diazotizable compounds recovered from the urine by the R-salt-coupling method following administration of 3- and 4-AAF, 3- and 7-iodo-2-AAF and 4-acetylaminobiphenyl gave evidence of a considerable production of "nitrite"-reacting metabolites other than 7-OH-2-AAF, but there is no correlation between the amounts formed and the degree of carcinogenicity of these compounds.

Dr. Paul N. Harris, Lilly Research Laboratories, Indianapolis, Ind.: Purified diets containing 2-AAF were given continuously to male and to female albino rats until liver-tumor development was certain or until other tumors jeopardized the life of the hosts. The rats were then killed for histologic examination and the cumulative tumor percentage was charted. Four variants of the basal diet (#4) differing only in a single constituent were used. Diet 5 contained 100 times as much riboflavine, diet 6 contained 100 times as much pyridoxine, diet 7 contained 0.002 mg. of biotin per gm., and diet 8 contained hydrogenated coconut oil instead of Primex.

Liver tumors were the most common in male rats on all diets and in female rats on diets 4 and 8. In female rats on diets 5, 6, and 7, tumors of the acoustic sebaceous gland were the most common, but liver tumors developed in 45 percent, 43 percent, and 56 percent of the effective total on these three diets. Tumors developed more slowly in female rats than in male rats.

Female rats on the control diet developed liver tumors most slowly, and those on diet 6 most rapidly. The rate for the other three diets was intermediate.

Liver-tumor development in male rats was slowest with diet 8 and most rapid with diet 6, but development with diets 4 and 5 was very little slower. The rate of tumor development with diet 7 was intermediate between that of diets 6 and 8.

Dr. James H. Leathem, Rutgers University, New Brunswick, N. J.: The paucity of information on the induction of hepatomas in dogs prompts me to bring our data to your attention. We have reported that 2-acetylaminofluorene in a purified diet induced hepatomas in 3 of 6 dogs. More recently 3 additional dogs have been studied but hepatomas were not induced. Nevertheless the carcinogen influenced the physiologic functions of the dog as nitrogen balance was reduced, plasma albumin-globulin ratios were decreased, and liver nitrogen and riboflavine were below normal levels.

The carcinogenic activity of 2-acetylaminofluorene is well documented. In our studies, 6 to 8 months' feeding resulted in liver involvement in 90 percent of male rats but in only 8 percent of the female rats. In contrast, when the carcinogen was fed to mice in laboratory chow, liver tumors failed to appear in 6 to 7 months in either sex. Increasing the carcinogen to 0.1 percent was not effective. However, when a semipurified diet (24% casein, 8% fat) was used liver tumors were induced in 10 of 13 male mice but in 0 of 12 mice in 6 months. Currently we are feeding the carcinogen to mice starting with the pregnant female and extending the feeding to the offspring into adulthood. Fertility has been good and F_3 litters have been obtained. Tumor incidence in F_1 mice was essentially as expected but liver tumors are appearing in F_2 mice despite the laboratory chow diet. Thiouracil is protective in the chow and also the semipurified diets.

Studies in our laboratory by Dr. Allison indicate that the urinary metabolites of 2-acetylaminofluorene are influenced by species and the levels of riboflavine and calcium pantothenate fed. Furthermore, the degree of conjugation of these amines cannot be estimated by determining the degree of diazotization before and after hydrolysis of urine.

Hepatomas in Choline Deficiency [1, 2]

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The occurrence of neoplasms in the liver and other tissues of rats as a result of prolonged choline deficiency was first reported from this laboratory in 1946 (*1*). Since that time, this work has been repeated and confirmed (*2–4*) and supported by the studies of choline deficiency in the chicken (*5, 6*).

Experimental Procedures and Results

Studies with Choline-Deficient Rats

The composition of two of the major types of diets that have been used in studies with chronic choline deficiency in the rat is given in table 1.

TABLE 1.—*Composition of diets**

Ingredients	Diet A	Diet B
Extracted peanut meal	30. 0	—
Extracted soybean meal or peanut meal	—	40. 0
Extracted casein	6. 0	—
Sucrose	39. 5	35. 6
Lard	19. 0	19. 0
Cod-liver oil	1. 0	1. 0
L-cystine	. 1	. 1
Salts No. 5†	4. 4	4. 4

*Vitamins in mg./kg. of diet: thiamine 2, pyridoxine 2, riboflavine 4, Ca-pantothenate 10, niacin 20, inositol 200, α-tocopherol 25, α-tocopherol acetate 25, and menadione 5.

†J. Nutrition 33: 155–168, 1947.

The peanut meal and casein were exhaustively extracted with hot methanol. Weanling rats, mainly of the AES strain weighing between 40 and 60 grams were placed in individual cages with wire floors. They were fed and watered daily and weighed weekly. Previous studies have shown that if these diets are not supplemented with minimal amounts of choline during the first few weeks, the rats will die with hemorrhage of the kidneys. Hence, the diets were initially supplemented with 0.10 percent

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 12, 1954.

[2] Published with the approval of the Director of Alabama Agricultural Experiment Station. This work was supported in part by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[3] The authors are indebted to Dr. William V. Hare, Pathology Department, University of Mississippi Medical School, for his assistance in interpretation of the microscopic material.

of choline chloride, which was reduced to 0 percent at the rate of 0.02-percent decrease each 2 weeks. In the early stages of the deficiency the livers were extremely fatty. They were enlarged and pale; histologic studies revealed that fat was distributed throughout the liver with fatty cyst-formation such as that described by Hartroft (*7, 8*). After a few weeks, the livers became cirrhotic; first there was only a rough surface; this was followed by development of a "hobnail" condition, where nodules varying from 2 mm. to 1 cm. or larger were found to cover the entire surface of the liver. After 6 months on the deficient diet, severe hobnail cirrhosis of the liver was consistently found. In the earlier stages of cirrhosis of the liver, histologic studies revealed that nearly all the liver tissue was fatty (fig. 1). As the cirrhosis progressed in severity, however, the amount of fat decreased, the amount of connective tissue increased, the normal architecture of the liver was lost, and pseudolobulation was pronounced. Numerous pseudolobules contained little or no fat (fig. 2). The cirrhotic livers contained neutral fat that was dissolved with the usual fat solvents, but could be demonstrated in frozen sections stained by the scarlet-red fat stain. There were also large deposits of a material that stained with the fat stain but they were not removed by the solvents. This material was identified as "ceroid."

Large regeneration nodules were consistently found in cirrhotic livers from rats that received the choline-deficient diet for 8 months or longer. These nodules varied in color and consistency from deep red to very pale (almost white), and from soft to moderately firm consistency.

There was considerable variation in the size of parenchymal cells that composed the regeneration nodules. In some cases, the cells were smaller than normal liver cells, but in most cases they were considerably larger with scattered cells several times normal size (fig. 3). It was not unusual to find liver cells with bizarre giant nuclei in hyperplastic nodules that were not neoplasms. Such nuclei were often hyperchromatic (figs. 4 and 5). Hyperplastic nodules frequently contained numerous mitotic figures (fig. 6).

Sections of cirrhotic livers from rats fed the choline-deficient diets for 12 months or longer varied in the amount and distribution of glycogen that was demonstrable by the periodic acid-Schiff technique. The unequal distribution of glycogen is illustrated in figure 7.

The gross appearance of large regeneration nodules in cirrhotic livers from choline-deficient rats is illustrated in figures 8 and 9. A significant number of regeneration nodules seemed to contain no portal triads and there appeared to be an absence of bile ducts. There was poor cord-formation (figs. 10, 11, and 12). The size of the nuclei was larger and there was a variation in the shape of the nuclei. The nuclei were hyperchromatic and there was an increase in the number of mitotic figures. When the cords were composed of several layers of cells and nuclear variation was pronounced, it was difficult to distinguish between hyperplasia and hepatocellular carcinoma. We have been conservative in classifying a number of such lesions as atypical regeneration nodules when there was any doubt whatever. The hepatocellular carcinomas had a more firm consistency and were generally lighter colored in gross than were the regeneration nodules. The tumors varied in size from 1 to 3½ cm. in diameter. The histology of one of the hepatocellular carcinomas that metastasized to the lungs is illustrated in figures 13 and 14. The cords appeared more like trabeculae of cells (2 to 10 layers thick) and there was considerable variation in structure in different parts of the tumor. In some areas the tumor cells formed ductlike structures. Slides of these areas stained by the PAS technique and by mucicarmine stains did not demonstrate the abundant mucin that is usually found in tumors of ductal origin. Only a minute amount of PAS-positive material was demonstrated and this might not have been mucin. Local invasion of the mesentery was frequently observed; small nodules in the mesentery appeared to be tumor tissue lying in lymph vessels or dilated venous channels. Positive metastases to the lungs were observed in 2 cases. The gross appearance of one of these tumors is shown in figure 15 and the microscopic appearance of the metastases to the lung is illustrated in figures 16 and 17. The histology of these tumors was no different from that of a number of tumors that did not metastasize.

Sections of tumors stained by the PAS technique revealed that there was not a uniform distribution of glycogen. In most of the tumors, there was less glycogen in the tumor tissue than in adjacent cirrhotic liver (fig. 18). In nearly all the cases, the distribution of glycogen was patchy. The hepatocellular carcinoma was the predominant tumor in rats fed the peanut meal plus casein diet (diet A). Bile-duct proliferation, especially a proliferation of ducts in interlobular tissue, cholangiofibrosis, and bile-duct cysts were frequently encountered. Because of the peculiar arrangement of the cells in some of the tumors, it was difficult to determine whether they originated from liver or bile-duct cells (figs. 19 and 20).

When diets containing peanut meal or soybean meal as the sole source of protein (diet B) were used, somewhat different results were obtained. Interlobular bile-duct proliferation, bile-duct cysts, and cholangiofibrosis (figs. 21 and 22) were consistently found, and the lesions of this nature were always much more extensive in animals fed diet B than in those fed diet A. In a significant number of animals, cholangiofibrosis was especially extensive and involved large parts of each lobe of the liver (fig. 23). These areas were light in color and very firm in consistency but were not necessarily raised above the surface of the liver. Microscopically, they were found to be composed of proliferating hyperplastic bile ducts. The ducts were atypical, cystic, and the cells were no longer like bile-duct cells (fig. 24). In many cases the cells were several rows thick. When abnormal nuclear variation and numerous mitotic figures were found in such lesions we classified them as atypical bile-duct hyperplasia, but with the feeling that some of these lesions might possibly be malignant. Some of these lesions were transplanted and after 3 months there were takes in 6 out of 8 rats that received transplants of one of the lesions. At present an attempt is being made to classify these lesions more accurately. A definite alveolar reticulum pattern was found in slides stained by the Bielschowsky technique. There was an accumulation of PAS-positive material in the lumen of the ducts which was probably mucin. A number of epithelial cells that make up the ductlike structures had PAS-positive material. This finding indicated that these lesions were of ductal origin. The predominant lesions in the livers of rats fed the diet B were of the bile-duct type. It is of interest that a simple change in diet could result in the production of a different type of liver lesion. Although a number of other types of tumors have been encountered in rats on chronic choline-deficiency studies, we have felt that the liver tumors were more significant.

Studies with Choline-Deficient Chickens

Under the conditions of experiments with choline deficiency in the chicken, liver cirrhosis was not consistently produced. In only 4 cases did the cirrhosis involve the entire liver. In most cases small areas of the liver were cirrhotic and these areas were more or less circumscribed. The livers were not uniformly fatty; some areas were extremely fatty but other areas of the same liver contained little or no fat. This may account for the pattern of the development of cirrhosis of the liver in the chicken. The chicken was markedly different from the rat with respect to fatty change and cirrhosis of the liver under conditions of these experiments. There was an increase in pigment, particularly in the liver, kidneys, spleen, and ovary of the chicken. The pigment was iron-positive as demonstrated by Turnbull's blue reaction, and was identified as hemosiderin (*6*). Regeneration nodules and liver tumors contained little or no pigment, but surrounding liver tissue contained large amounts of iron pigment. A significant incidence of tumors of the liver were produced in choline-deficient chickens, but in contrast to the rat the tumors of the liver in the chicken were seldom associated with cirrhosis of the liver and were predominately of ductal origin (cholangiocarcinoma). The typical gross appearance of tumors of the liver of choline-deficient chickens is illustrated in figure 25. The microscopic appearance of the tumors varied considerably but extensive studies using special stains proved them to be cholangiocarcinomas. In some cases the tumor cells were arranged in solid masses as shown in figure 26 but in most cases the ductal origin of these tumors was very apparent (fig. 27). The mucous material in the acinar structures was stainable with

both PAS and mucicarmine stains. Sarcomas and fibrosarcomas were the tumors most frequently found in the choline-deficient chickens. A variety of sarcomas (rhabdomyosarcoma, reticulum-cell sarcoma, leiomyosarcoma, and liposarcoma) were seen. Since spontaneous liver tumors and liposarcoma are very rare in chickens, the development of these tumors in the chicken was considered significant. Although liposarcomas were found in only two chickens, in both cases the chickens received a diet with peanut meal or soybean meal as the sole source of protein. The gross appearance of one of the liposarcomas is illustrated in figure 28. Metastases to the heart and lung from this tumor are illustrated in figure 29. The tumor was composed of small fusiform cells with considerable fibrous connective tissue (fig. 30). Frozen sections stained with scarlet red revealed the typical features of liposarcoma (fig. 31).

Several types of kidney tumors were found: myxofibromas, embryonal nephromas, reticulum-cell sarcomas, tumors resembling teratoma, and typical and atypical Wilm's tumors.

Studies with Choline-Deficient Dogs

Cirrhosis of the liver was a consistent finding in choline-deficient dogs. Large regeneration nodules were found in 10 out of 26 chronic choline-deficient dogs (fig. 32). Bile-duct proliferation was observed in 9 out of 26. In most cases this proliferation was confined to the interlobular tissue (fig. 33). Duodenal ulcer was observed in 13 out of 26 dogs. Jaundice and ascites were commonly found.

Heart and Vascular Lesions

Heart and vascular lesions were observed in chronic choline-deficient rats, chickens, and dogs (25 to 50%). The blood vessels of the lungs were most commonly involved. Other locations according to incidence were endocardium, coronary vessels, aorta, pancreatic, splenic, and liver vessels. In numerous cases the lesions were apparent in the gross.

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PLATE 123

FIGURE 1.—Cirrhosis of the liver of a choline-deficient rat. In early stages each pseudolobule contained fat. Hematoxylin and eosin. × 275

FIGURE 2.—Cirrhosis of the liver of a choline-deficient rat. There is variation in the fat content of different lobules. Hematoxylin and eosin. × 300

FIGURE 3.—Regeneration nodule in cirrhotic liver of a choline-deficient rat. There is an increase in number of cells. The nuclei vary in size. Hematoxylin and eosin. × 300

PLATE 124

FIGURE 4.—Large cells from an area of regeneration in a cirrhotic liver of a choline-deficient rat. The cells are several times normal size and the nuclei are hyperchromatic. Hematoxylin and eosin. × 1,000

FIGURE 5.—Large cell from cirrhotic liver of a choline-deficient rat. The nucleus is abnormal in shape. Hematoxylin and eosin. × 875

FIGURE 6.—Mitotic figure in an area of regeneration in a cirrhotic liver of a choline-deficient rat. Hematoxylin and eosin. × 850

PLATE 125

FIGURE 7.—Photomicrograph illustrating variation in the amount of glycogen contained in different pseudolobules of a cirrhotic liver from a choline-deficient rat. Periodic acid-Schiff. × 100

FIGURE 8.—Photograph of a cirrhotic liver from a choline-deficient rat. Note the large regeneration nodules.

FIGURE 9.—Photograph of a cirrhotic liver from a choline-deficient rat. Note the large nodules.

PLATE 126

FIGURE 10.—Photomicrograph of an area of atypical cells from a hepatoma of the liver of a choline-deficient rat. The nuclei are hyperchromatic and vary in size and shape. Mitotic figures are present. Hematoxylin and eosin. × 550

FIGURE 11.—Atypical regeneration nodule from cirrhotic liver of a choline-deficient rat. Liver cells formed ductlike structures. Hematoxylin and eosin. × 230

FIGURE 12.—Hepatoma of the liver of a choline-deficient rat. The cells are arranged in cords several layers thick. Hematoxylin and eosin. × 230

PLATE 127

FIGURE 13.—Photomicrograph of an area of a hepatocellular carcinoma of the liver of a choline-deficient rat. Note the peculiar arrangement of the cells and numerous mitotic figures. Hematoxylin and eosin. × 230

FIGURE 14.—Photomicrograph of a different area of the same tumor shown in figure 13. There is no specific arrangement of the cells. Hematoxylin and eosin. × 245

PLATE 128

FIGURE 15.—Photograph of liver of a choline-deficient rat illustrating the gross appearance of one of the hepatocellular carcinomas—metastases to the lung were found in this case.

FIGURE 16.—Photomicrograph of an embolus of tumor cells in a blood vessel in the lung of choline-deficient rat No. 14067. Hematoxylin and eosin. × 275

FIGURE 17.—Photomicrograph of a pulmonary metastasis from a liver tumor of a choline-deficient rat. Hematoxylin and eosin. × 310

PLATE 129

FIGURE 18.—Photomicrograph illustrating an area of hepatoma that contains less glycogen than adjacent cirrhotic liver. Periodic acid-Schiff. × 150

FIGURES 19 and 20.—Photomicrographs of 2 areas of a liver tumor which are of questionable origin. There are formations of cells which appear to be cystic, ductlike structures (fig. 19), and a giant cell is seen in figure 20. Hematoxylin and eosin. × 200

FIGURE 21.—Cholangiofibrosis in the liver of a choline-deficient rat. Hematoxylin and eosin. × 100

FIGURE 22.—Photomicrograph of an area of cholangiofibrosis in the liver of a choline-deficient rat to illustrate the cellular structure. Hematoxylin and eosin. × 300

PLATE 130

FIGURE 23.—Photograph of slices of liver of a choline-deficient rat fed diet B to show extent of cholangiofibrosis. Only small areas of parenchymal cells remain. Lee Brown's modification of Mallory's stain. × 10

FIGURE 24.—Photomicrograph of liver (area indicated in fig. 23) to illustrate the cellular nature of a lesion classified as atypical bile-duct proliferation. Hematoxylin and eosin. × 230

PLATE 131

FIGURE 25.—Photograph illustrating gross appearance of liver tumors in liver of a choline-deficient chicken.

FIGURE 26.—Photomicrograph of a cholangiocarcinoma of the liver of a choline-deficient chicken. An area with cells packed solid. Hematoxylin and eosin. × 400

FIGURE 27.—Photomicrograph of a cholangiocarcinoma of the liver of a choline-deficient chicken. The ductal origin of this tumor is apparent. Hematoxylin and eosin. × 235

PLATE 132

FIGURE 28.—Photograph to illustrate the gross appearance of a liposarcoma in a choline-deficient chicken.

FIGURE 29.—Photograph to illustrate gross appearance of metastases of the tumor shown in figure 28 to the heart and lung.

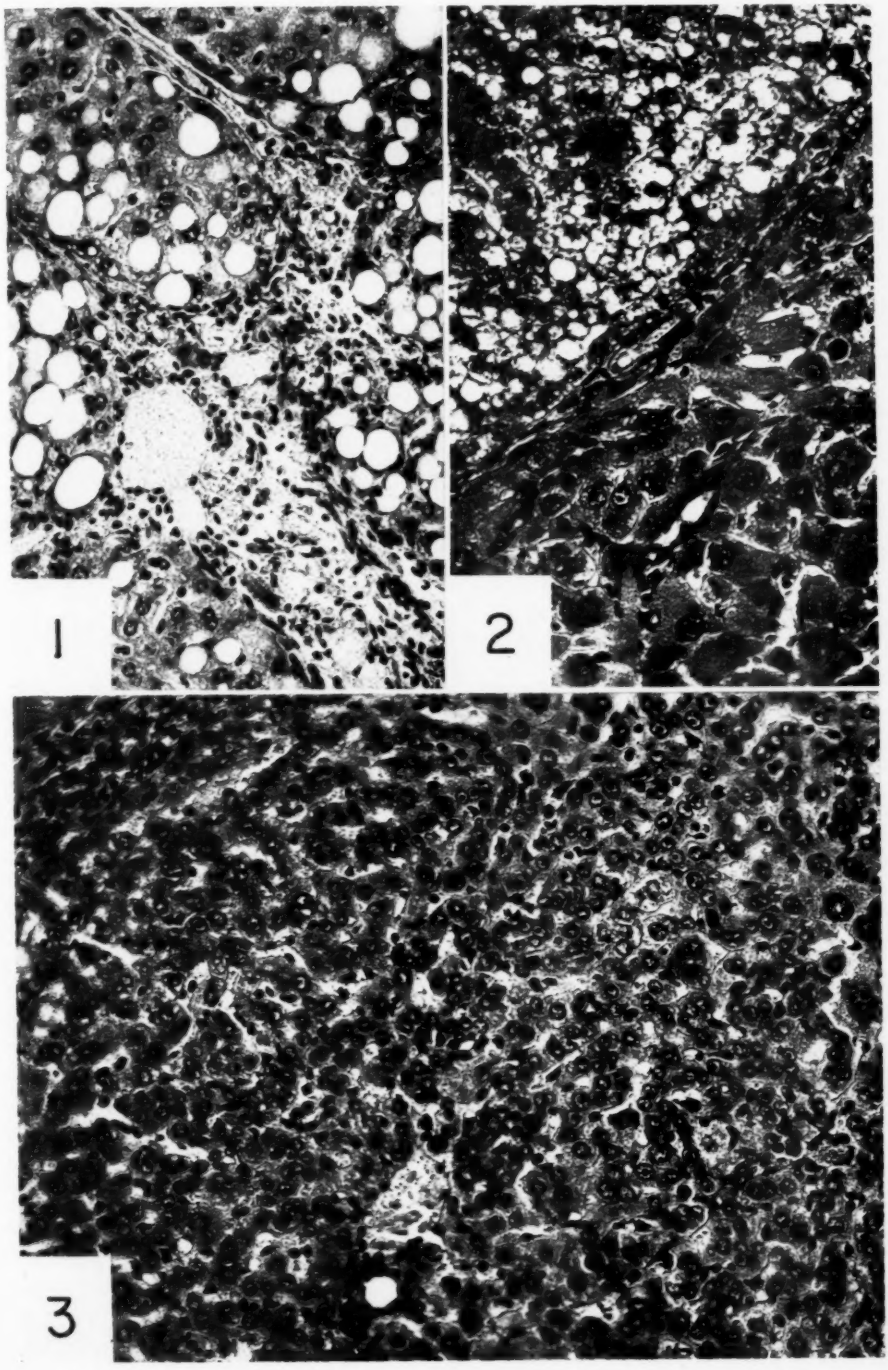
FIGURE 30.—Photomicrograph of liposarcoma from a choline-deficient chicken (fig. 28). Hematoxylin and eosin. × 150

FIGURE 31.—Photomicrograph of a frozen section of the tumor shown in figure 28. Frozen section stained with scarlet red and hematoxylin. × 1,400

PLATE 133

FIGURE 32.—Photograph of a slice of cirrhotic liver of a choline-deficient dog to show extent of cirrhosis. Hematoxylin and eosin. × 8

FIGURE 33.—Photomicrograph of a section of liver shown in figure 32 showing bile-duct proliferation in the interlobular area. Hematoxylin and eosin. × 250



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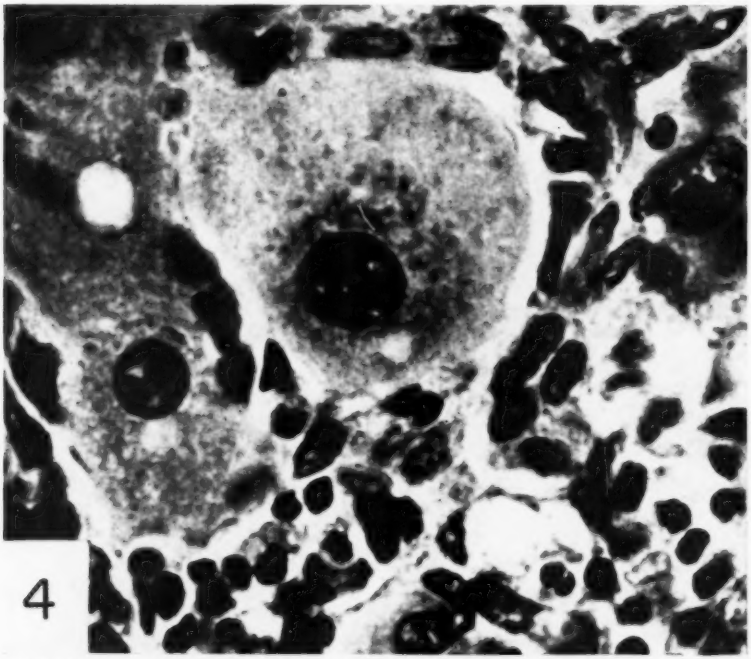

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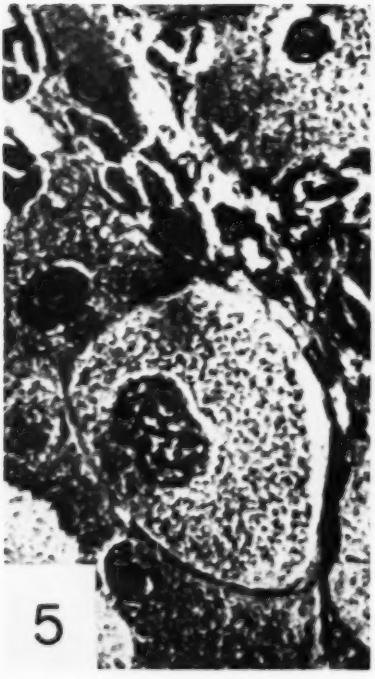

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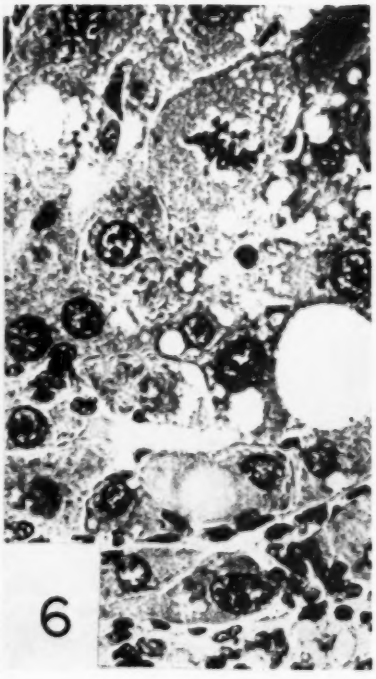

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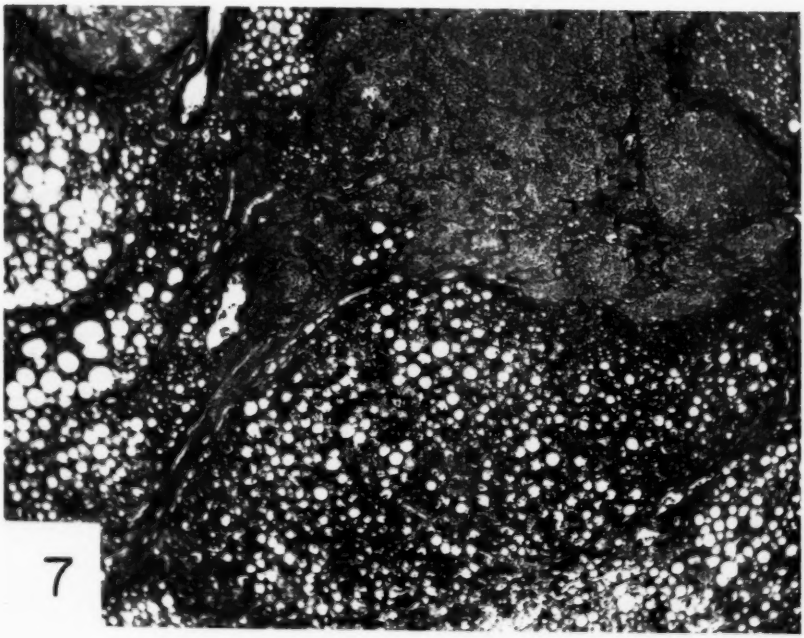

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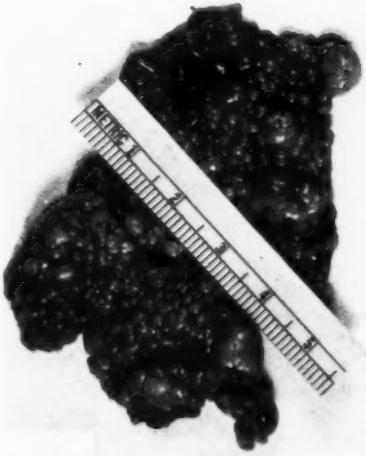

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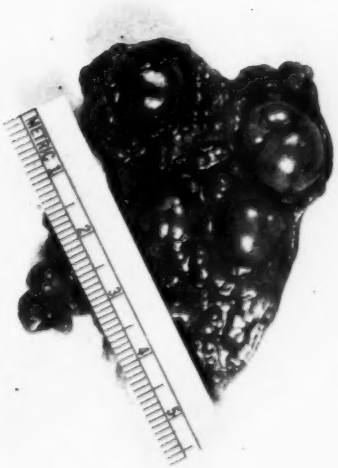

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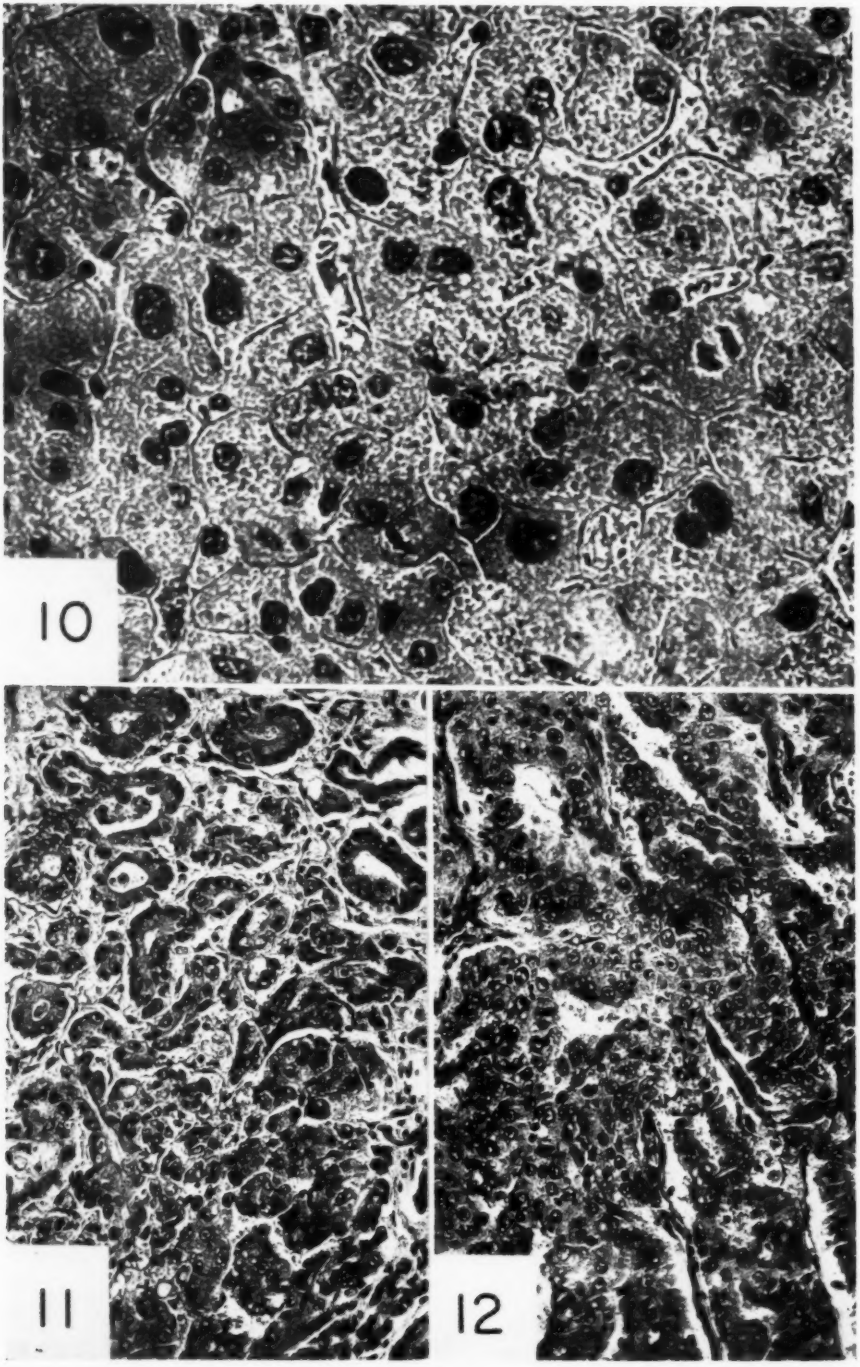
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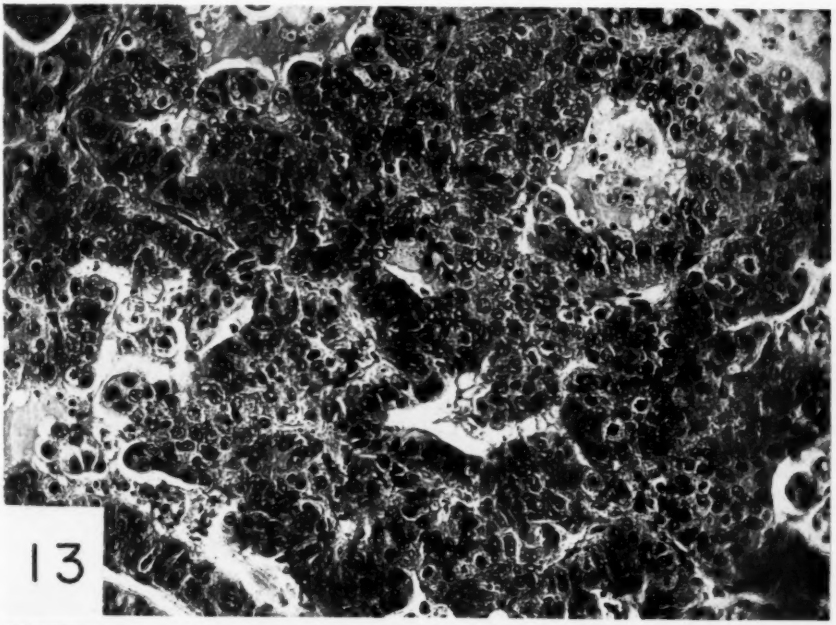

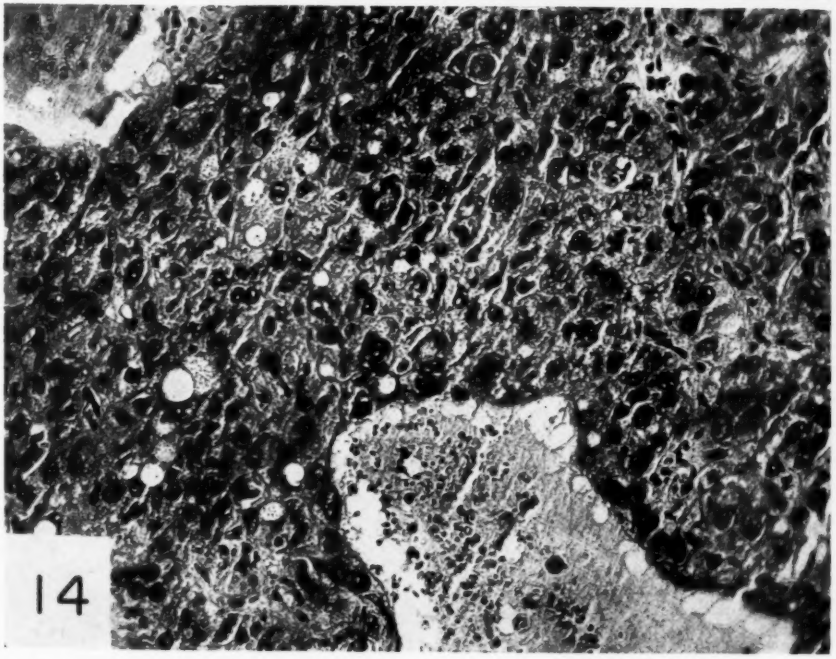

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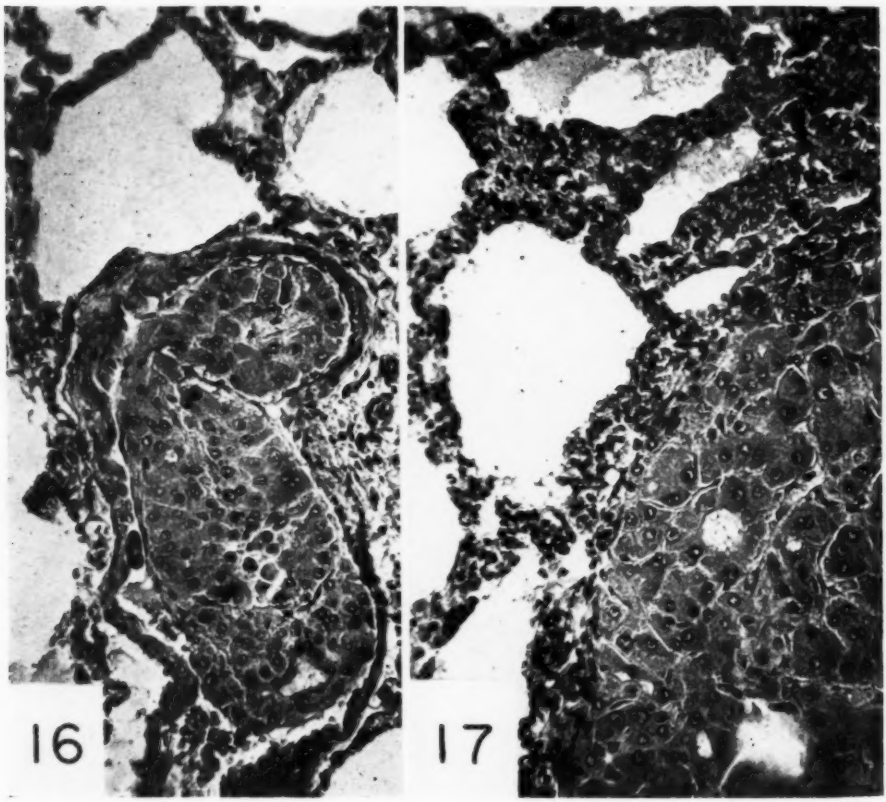

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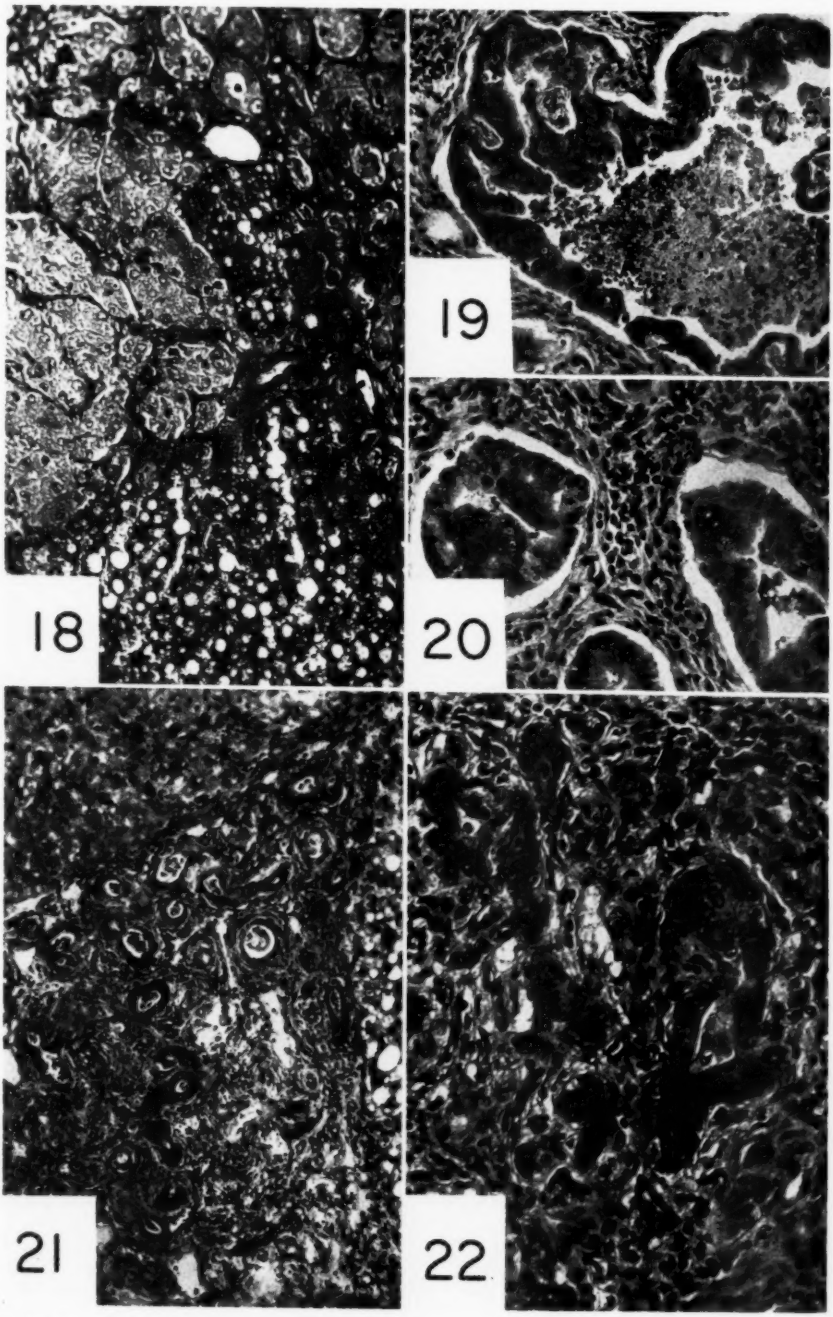
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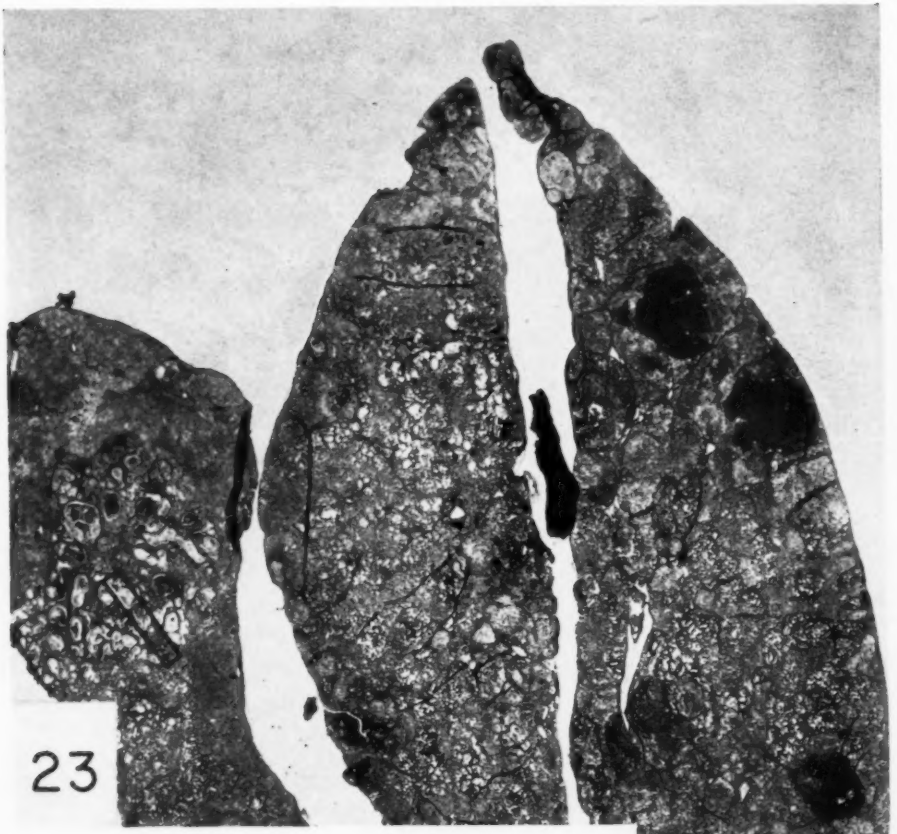
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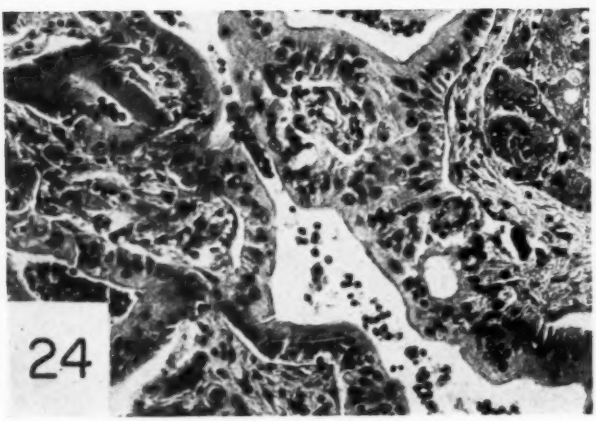
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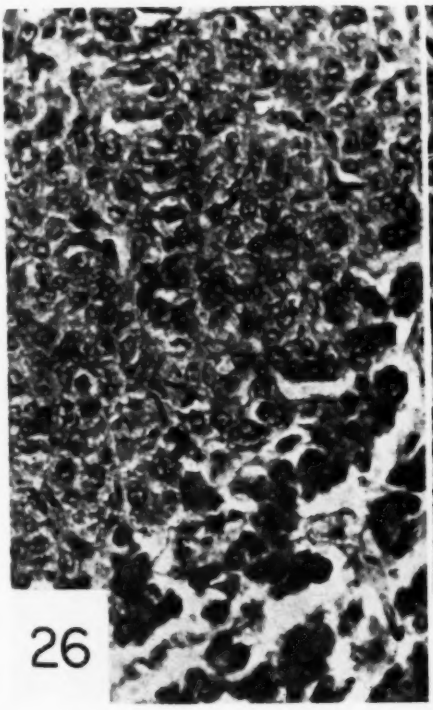
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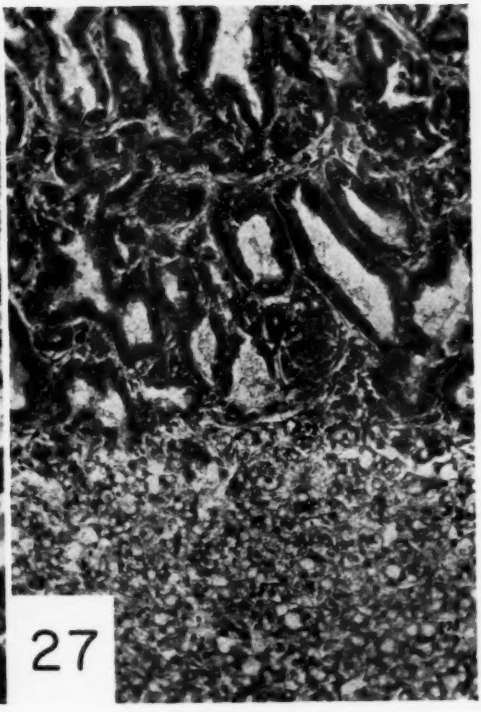
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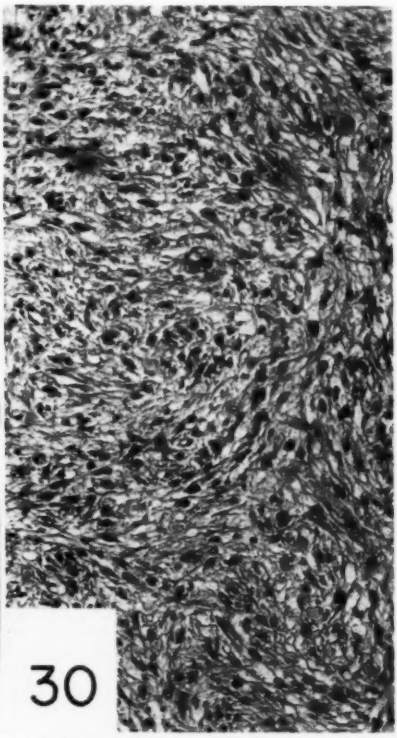

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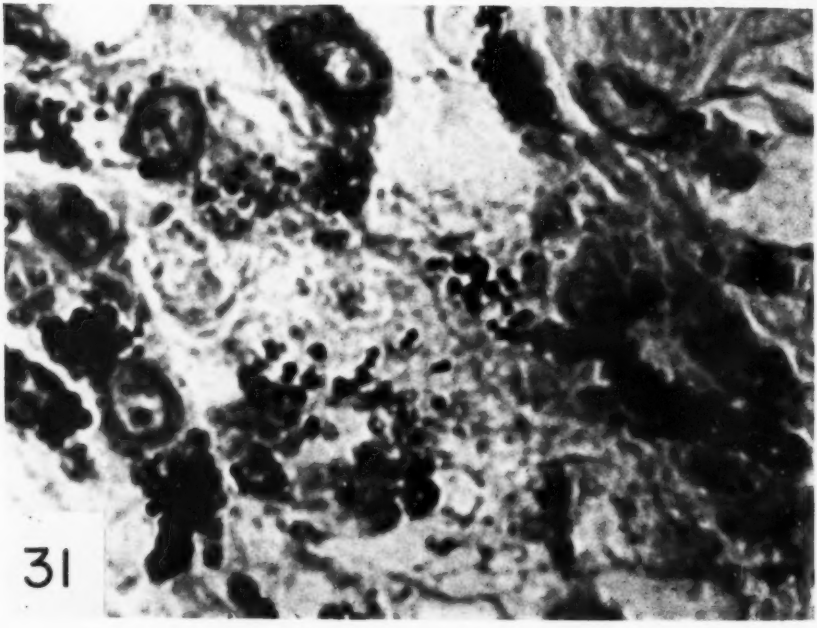

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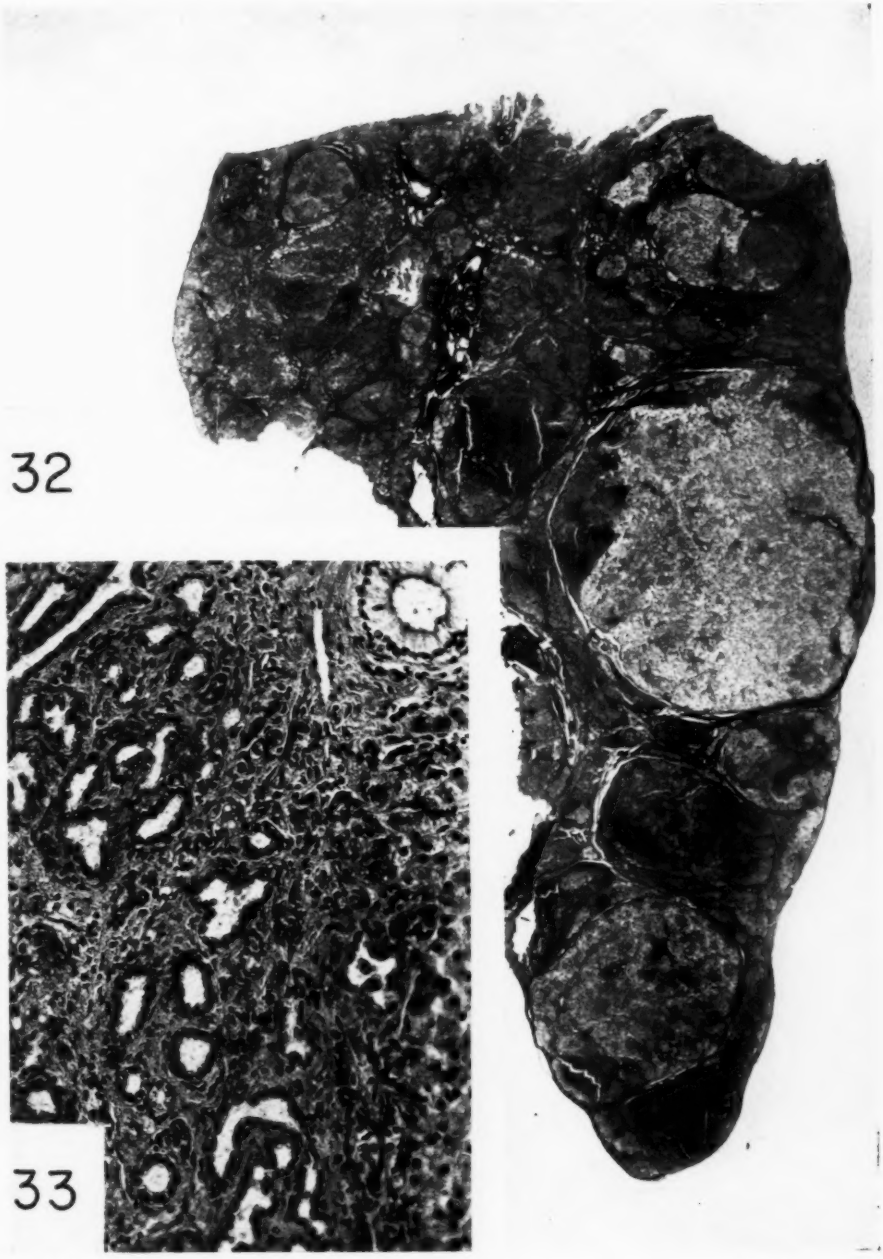
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Dr. W. E. Cornatzer, University of North Dakota, Grand Forks, N. Dak.: I have enjoyed Dr. Copeland's review of the histologic changes that occur in the production of experimental hepatomas in choline deficiency. In our laboratory, we have been carrying out an extensive study of the phospholipide changes that occur in various cellular fractions of the liver in choline deficiency. In a choline deficiency, the greatest decrease of lipide P was observed in the lecithin mitochondria fraction of the liver.

I would like to confirm Dr. Copeland's observation of the production of hepatomas in rats maintained on a diet low in choline. Attempts to compare the rate of incorporation of P^{32} into the liver phospholipides of rats maintained on a 5-percent casein–5-percent fat diet at various time intervals (64, 96, 114, 164, 185, 206, 249, and 297 days) in the production of hepatomas have been made. The rats were injected with P^{32}; and after 6 hours the acid-soluble P, lipide P and nitrogen were determined. From these results and radioactivity measurements, the relative specific activities of the phospholipides were calculated.

Hepatomas were produced in the animals maintained on the diet for 185 days. The phospholipide synthesis was not altered during the production of hepatomas. The phospholipide synthesis remains rather constant, even though there is a total decrease in the lecithin P in the liver as the choline deficiency progresses. The phospholipide synthesis, as measured by the relative specific activity, was increased in those animals in which hepatomas were observed. The phospholipide P/N ratio remained unchanged during the production of hepatomas and in the tumor itself. At present, lipide analysis of the mitochondria and other cellular fractions of experimental hepatoma are being investigated.

Dr. H. I. Firminger, University of Kansas Medical Center, Kansas City, Kans.: This presentation of Dr. Copeland's illustrates very nicely the series of morphologic changes which take place in the liver of rats during the development of hepatocellular carcinoma of the liver. The changes which he has illustrated are entirely similar to those that occur when carcinogens are administered as I showed yesterday, except for the greatly increased amount of cirrhosis which is to be expected on the diet which Dr. Copeland employed. There is one thing which seems unaccounted for on the basis of choline deficiency alone. According to the work of White and Edwards (J. Nat. Cancer Inst. 3: 43–59, 1942), the bile-duct proliferation with cyst-formation and cholangiofibrosis occurred only with the addition of carcinogen to their diet. The cyst-formation and cholangiofibrosis in these animals of Dr. Copeland's is quite striking and although I do not believe he has illustrated a fully developed cholangiocarcinoma in any of his rats, he certainly has their precursors and I feel it is merely a matter of longer time interval or more animals before true cholangiocarcinoma will be found.

The effect of the diet on the bile ducts is also borne out by the cholangiocarcinoma developing in the chicken; assuming, of course, that liver tumors do not develop spontaneously in chickens. This tumor in the chicken developed without cirrhosis and further substantiates the point I tried to make yesterday, that cirrhosis in itself is not necessary for the development of liver tumors and that some additional factor or factors must be incriminated. Of course, the tumor which we saw in the chicken was a cholangiocarcinoma and in human beings such tumors are not correlated with cirrhosis as are the hepatomas of the liver-cell type.

I wonder whether Dr. Copeland can completely assure us that there was no carcinogen contained in some of the peanut meal or other constituents in the diet in view of these additional changes. Has he run any other test of the carcinogenicity of these materials? I hope he can give us such assurance because I like peanuts.

Dr. D. H. Copeland, Alabama Polytechnic Institute, Auburn, Ala.: Pending further studies of transplants from bile-duct lesions in the rat, we are classifying them simply as atypical cholangiofibrosis. We believe there is reasonable assurance that the peanut meal and other diet ingredients contain no recognized carcinogen. Similar lesions were produced when the peanut meal was replaced with soybean meal. Moreover the lesions were produced only when the diet was deficient in choline and related factors.

Dr. Charles Berman, Consolidated Main Reef Mine Hospital, Maraisburg, Transvaal, South Africa: I wish to congratulate the authors on the presentation of such an excel lent paper, and ask the following questions:

1) In the tumors of the liver produced in chickens on choline-deficient diets, iron pigment was found in the unaffected liver cells. This was absent in the tumorous livers of the rats.

Among the Bantu of South Africa iron pigment is commonly encountered in adult livers, especially with cirrhosis. There are today two schools of thought in South Africa concerning the origin of the pigment. *a*) This school is of the opinion that the iron pigment is derived from the diet and from the iron pots which the Bantu people use to cook their food and brew their beer. *b*) This school, on the other hand, contends that the iron pigment is derived from cellular material within the mitochondria of the liver cell in malnourished Bantu adults.

Is the food used for the chickens prepared in iron vessels, and is this not the case with the food of the rats?

2) Dr. J. G. Campbell of Edinburgh had some years ago described spontaneous liver cancers in Aylesbury ducks in England. Recently, Dr. Campbell spent some time in Siam where he studied similar liver tumors occurring spontaneously in indigenous ducks in that country (personal communication to Dr. Berman), and is now back in Edinburgh where his is attempting to induce liver tumors in ducks with Senecio alkaloids. Has Dr. Copeland done any experimental feeding of ducks in this country to test their susceptibility to liver cancer?

Dr. Copeland: Iron pots were not used in extraction of the peanut meal and casein. We use copper steam jacket continuous extraction units which eliminates this source of the iron pigments. It should be pointed out that the diets of both the deficient and the control animals contained adequate iron salt in the salt mixture. We have not used ducks in any of our experiments.

Dr. J. N. P. Davies, Makerere College Medical School, Kampala, Uganda, East Africa: Because your observations may be of relevance to the problems of human pathology of hepatomas, could you say if you observed differences between the two sexes, in rats and chickens, as regards their susceptibility to hepatoma formation?

Do the liver cells in the hyperplastic nodules contain fat?

When hepatomas arise in the livers of your experimental animals is there fat present in the liver cells as well as a degree of liver fibrosis?

Dr. Copeland: There was no significant sex difference with relation to the development of tumors in rats and chickens. In some cases the cells in the hyperplastic nodules contained fat, but in many cases little or no fat was found. The areas around the nodules frequently contained large amounts of fat. In the case of the rat the development of liver tumors was always associated with fatty livers and cirrhosis of the liver, but in the case of the chicken some of the tumors developed in livers that were not fatty or cirrhotic. The majority of tumors of the liver in the chicken, however, did develop in fatty livers.

The Effects of Compounds Related to Choline on Azo-Dye Carcinogenesis[1]

C. J. KENSLER, E. BIERMAN, *and* G. CONDOURIS, *Department of Pharmacology, Cornell University Medical College, New York, N. Y., and Arthur D. Little, Inc., Cambridge, Mass.*

The possible relationship between the production of hepatic tumors by azo dyes and those reported to occur in rats maintained on low choline diets (*1*) has been discussed previously (*2*). Although choline supplements *per se* did not reduce tumor incidence obtained with nonprotective diets, the observation that choline oxidase activity was markedly reduced suggested that betaine might reduce the incidence. Betaine, however, possibly because betaine-homocysteine transmethylase activity was also reduced in azo-dye-treated livers, was ineffective.

This study has been extended to include dimethylthetin (sulfur analogue of betaine), glycine, aminoethanol and dimethylaminoethanol. Dimethylthetin, which *in vitro* with rat liver homogenates is a much more effective methyl donor than betaine in the methylation of homocysteine, has been found in two experiments to afford partial protection against azo-dye carcinogenesis; however, not to the same extent as riboflavine-casein supplements. Glycine afforded no significant protection.

In our most recent experiment summarized in table 1, ethanolamine, but not dimethylaminoethanol, supplements have been found to markedly reduce tumor incidence as a result of azo-dye feeding. Unlike the riboflavine-casein-type protection (*3*), the rats supplemented with either ethanolamine or dimethylaminoethanol failed to grow, and food consumption was lower than in the rats fed the basal ration containing the dye. Aminoethanol afforded protection; whereas, dimethylaminoethanol did not, although there was no significant difference in growth or food consumption between the two groups. As the animals fed the diet supplemented with dimethylaminoethanol and those fed the diet supplemented with aminoethanol consumed less dye than those

TABLE 1.—*Inhibition of azo-dye carcinogenesis by ethanolamine*

Diet*	Rats surviving more than 100 days (*number*)	Liver tumors by 155 days (*percent*)	Food consumption (*gm./rat/day average*)
5. DMB	19	67	11. 1
17. DMB+AE	14	8	8. 6
18. DMB+AE+DMT	16	8	7. 9
19. DMB+DMAE	12	77	6. 7
20. DMB+DMAE+DMT	17	63	6. 9
21. DMB+OH-Meth	19	35	10. 0

*DMB=*N*, *N*-dimethyl-*p*-aminoazobenzene; AE=aminoethanol; DMT=dimethylthetin; DMAE=dimethylaminoethanol; OH-Meth.=Hydroxy analogue of methiomine.

[1] Presented at the Conference on Experimental Hepatomas, Harriman, N. Y., October 12, 1954.

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fed the basal diet—carcinogen controls, it is possible that the difference in tumor incidence is due to a pro-carcinogenic action of dimethylaminoethanol.

In view of the report of Dent and associates (*4*) of the excretion of large quantities of ethanolamine in the urine of a patient with primary hepatoma and the report of Reid, Landefeld, and Simpson (*5*) that the ethanolamine portion of the choline molecule isolated from rat liver tumors is probably formed by a different pathway than that in liver choline, it appears that a more intensive study of the role played by ethanolamine in hepatocarcinogenesis is warranted.

References

(*1*) Copeland, D. H., and Salmon, W. D.: Occurrence of neoplasms in liver, lungs, and other tissues of rats as a result of prolonged choline deficiency. Am. J. Path. 22: 1059–1079, 1946.

(*2*) Kensler, C. J.: Relationship of nutritional factors to the chemical induction of tumors. Texas Rep. Biol. & Med. 10: 1006–1022, 1952.

(*3*) Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P.: Partial protection of rats by riboflavin with casein against liver cancer caused by dimethylaminoazobenzene. Science 93: 308–310, 1941.

(*4*) Dent, C. E., Fowler, D. I., and Walshe, J. M.: Ethanolaminuria, an obscure metabolic defect associated with a case of primary hepatoma. Biochem. J. 48: xiii–xiv, 1951.

(*5*) Reid, J. C., Landefeld, M. O., and Simpson, J. L.: Metabolism of L-histidine-2-C^{14} in the normal and the hepatoma-bearing rat. J. Nat. Cancer Inst. 12: 929–936, 1952.

Biochemical Investigations on Hepatic Carcinogenesis[1,2]

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McArdle Memorial Laboratory for Cancer Research, Medical School, University of Wisconsin, Madison, Wis.

In preparing this discussion we attempted to bring together certain of the data on the induction of liver tumors by various chemical compounds. In so doing, we have tried to examine the biochemical similarities and differences in the processes induced by these agents and to sort out the changes which may be of importance in the induction of liver tumors from those which may be only secondary factors. However, these purposes have to be pursued in terms of data which are still meager in many important areas. Many further studies are needed before one can outline the details of the carcinogenic process induced by any one of these compounds.

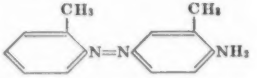
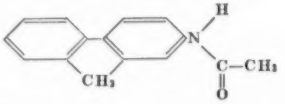
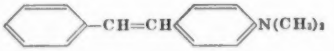
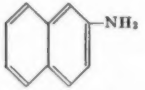
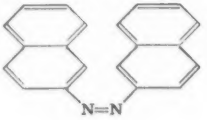
As a general review, the chemical agents for which hepatocarcinogenic activity has been clearly established are listed in table 1; the species of animals known to be susceptible to these carcinogens are also indicated. The data on these compounds through 1947 have been compiled by Hartwell (*1*), and we (*2*) have recently reviewed the studies on azo-dye carcinogenesis. Where applicable, these sources will be cited and may be consulted for the original literature. The aminoazo dyes have received more attention than any other compound or group of compounds, possibly because they were the first known chemical carcinogens for the liver. 4-Dimethylaminoazobenzene (DAB) will induce malignant tumors (hepatomas and cholangiomas) in the livers of essentially all the rats fed the dye as 0.05 to 0.06 percent of a low riboflavine, but otherwise nutritionally adequate, diet for 3½ months or longer. DAB is also carcinogenic for mouse liver but less active. Liver tumors have not resulted when DAB was fed for long periods to dogs, cotton rats, guinea pigs, rabbits, hamsters, chipmunks, or chickens, although tumors of the urinary bladder were found in two of five dogs fed the compound up to 5 years (*1–3*). On the other hand, 2′,3-dimethyl-4-aminoazobenzene (*o*-aminoazotoluene) will induce a high incidence of liver tumors in mice when administered orally or by subcutaneous injection, but it is a relatively weak carcinogen for rat liver (*1, 2, 4*). In our experience, only about 25 percent of the rats fed 0.06 percent of *o*-aminoazotoluene for 16 months developed liver tumors (*5*). Nelson and Woodard (*3*) found tumors in the livers of two of five dogs fed this dye for 30 to 62 months; tumors were found in the urinary bladders of two other dogs.

2-Acetylaminofluorene (AAF) and certain related compounds (*1, 6–10*) induce a high incidence of liver tumors in rats when fed for 4 to 5 months as 0.025 to 0.05 percent of the diet. Mice, cats, dogs, and hamsters (see below) also develop some liver tumors when fed AAF, but are much less susceptible than rats (*1, 9, 11–13*). 4-Aminostilbene and certain of its derivatives were found by Haddow and his associates (*14*) to induce

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 12, 1954.

[2] The work of the authors in this field has been supported by grant C-355 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, INSTR grant No. 71B from the American Cancer Society, and a grant from the Alexander and Margaret Stewart Trust Fund.

TABLE 1.—*Compounds with hepatocarcinogenic activity*

Compound	Formula	Species*
Aminoazo dyes		
4-Dimethylaminoazobenzene		Rat, mouse
2′,3-Dimethyl-4-aminoazobenzene (*o*-aminoazotoluene)		Mouse, rat, dog
2-Acetylaminofluorene		Rat, mouse, dog, cat, hamster
4-Dimethylaminostilbene		Rat
Carbon tetrachloride		Mouse
β-Naphthylamine		Mouse
2,2′-Azonaphthalene		Mouse

3,4,5,6-Dibenzcarbazole		Mouse
Miscellaneous		Rat
KNH_4Se, thioacetamide, thiourea, benzidine, ethionine, 1,2-benzanthracene, senecio alkaloids, tannic acid		

*Where more than one species is listed, the first named is the most susceptible.

liver tumors in rats when the compounds are either fed or injected. Although the total amount of the aminostilbenes required for the induction of tumors is relatively low, the toxicity of the compounds limits the doses which can be administered, so that long latent periods are required. Mice given subcutaneous injections of these compounds did not develop liver tumors. Unlike the aminoazo dyes which are relatively specific as liver carcinogens, 2-acetylaminofluorene, 4-aminostilbene, and related compounds are also carcinogenic for other tissues—especially the mammary gland, ear duct, and small intestinal epithelium, but this aspect of their activities will not be discussed here.

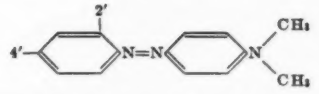
Carbon tetrachloride and chloroform induce a high incidence of hepatomas in mice treated repeatedly by stomach tube. While the prolonged administration of carbon tetrachloride eventually induces a severe cirrhosis in the livers of rats, tumor induction has not been achieved (*1*). 2,2′-Azonaphthalene, 3,4,5,6-dibenzcarbazole, and β-naphthylamine likewise induce tumors in the livers of mice (*1*, *15*, *16*). Finally, a number of other compounds—ammonium potassium selenide (*1*), benzidine (*17*), senecio alkaloids (*18*), ethionine (*19*), thioacetamide (*20*), thiourea (*20*), and tannic acid (*21*, *22*)—have been reported to induce liver tumors in a small to moderate percentage of the rats treated.

It has been a general observation that at least some degree of liver damage precedes and accompanies the induction of hepatic tumors by these compounds. While it is difficult to determine whether or not these changes are directly related to the induction of tumors, it is noteworthy that the more carcinogenic compounds generally cause the most severe alterations in liver histology. Furthermore, little or no liver damage is usually found in the species resistant to the tumor-inducing activity of these compounds. These changes have been described in detail by others [see, for instance, (*23–27*)] and will not be considered here.

The gross and microscopic alterations are accompanied by, and are undoubtedly the result of, biochemical changes. In general, the biochemical alterations seem to be relatively similar whether a carcinogenic azo dye or AAF is administered (*28–34*). Thus, in both cases the deoxyribonucleic-acid content of the liver cells remains approximately normal; however, since the cells become smaller than normal, the concentration of this nucleic acid per gram of liver increases up to twice the normal value. At the same time the amounts of mitochondrial and microsomal protein and ribonucleic acid and of certain enzyme activities decrease. In the case of the mitochondria this is due in large part to a decreased number of these particles per cell (*28*, *33–37*). Of particular interest to us have been the observations that the cells of the tumors induced by the aminoazo dyes and by AAF differ from normal liver cells in a similar, but more exaggerated, manner than do the livers of rats fed these carcinogens. This fact suggests that at least some of the early changes in the liver cells may be related to the induction of the tumor cells. Perhaps, indeed, many of the cells in the livers of rats fed these carcinogens for periods short of tumor formation are what might be termed "part tumor" cells— *i.e.*, cells that are altered from normal in such a manner that with the proper additional stimulus they could be converted to cancer cells. However, the extensive alterations in the histology of the liver during the precancerous phase seriously complicate the interpretation of this type of data.

In the cases examined (primarily the aminoazo dyes and AAF) the carcinogens are extensively metabolized, especially by the liver. The metabolism of DAB has been considered extensively elsewhere (*2*) and is summarized briefly in text-figure 1. As indicated, one of the major metabolic reactions is *N*-demethylation; this leads first to the *N*-monomethyl derivative, 4-monomethylaminoazobenzene (MAB), which is as active carcinogenically as DAB and which is in equilibrium with it *in vivo*. MAB is the only known carcinogenic metabolite of DAB. The demethylation then proceeds essentially irreversibly to 4-aminoazobenzene (AB). However, traces of *N*-monomethyl dye can be observed under appropriate conditions when AB is fed, and appreciable quantities when its 3′-methyl or 4′-fluoro derivatives are fed (*38*). This finding is of considerable interest since, unlike AB, 3′-methyl- and 4′-fluoro-4-aminoazobenzene are both carcinogenic though relatively weak as compared to their *N*-methyl deriva-

tives. Their activities can be correlated with the extent of their conversions to the *N*-monomethyl derivatives *in vivo*. The major share of the ingested dye is also reductively cleaved at the azo linkage and hydroxylated, especially in the 4′-position, at some stage in its metabolism. The sequence of these reactions *in vivo* has not been established, although *N*-demethylation apparently occurs first. All these reactions lead to noncarcinogenic compounds. Approximately 60 percent of the administered dye has been accounted for as known compounds, principally conjugates of *p*-aminophenol and *p*-phenylenediamine, in the urine. Studies with DAB labeled with C^{14} in the aromatic rings showed that another 10 percent of the dye is excreted in the urine as unidentified metabolites and that about 20 percent of the administered C^{14} is excreted in the feces (*39*). A small percentage of the dye is not extensively degraded and is combined with certain liver proteins through a covalent linkage. As will be discussed later, it is this fraction of the ingested dye which appears to us to be of importance in the induction of liver tumors.

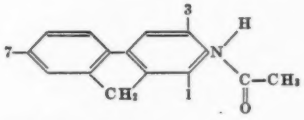


1) *N*-Demethylation: $-N(CH_3)_2 \rightleftharpoons -NHCH_3 \rightarrow -NH_2$
2) Reduction of the azo linkage: $-N=N- \rightarrow -NH_2 + H_2N-$
3) Hydroxylation, primarily at 4′-position; lesser amount at 2′-position.
4) Binding of azo-dye metabolites to liver protein.

TEXT-FIGURE 1.—Reactions involved in the metabolism of 4-dimethylaminoazobenzene in the rat.

Morris, Weisburger, and Weisburger (*40*) have found that AAF is deacetylated to 2-aminofluorene in the rat (text-fig. 2); this compound is also a strong carcinogen (*1*, *7*, *9*, *10*). Hydroxylation occurs principally in the 7-position (*para* to the amino group as in DAB) (*41–43*) and in small amounts at positions 1 and 3, *ortho* to the amino group (*42*, *43*). The known urinary and fecal metabolites account for only about one half of the administered compound. As in the case of DAB, small amounts of AAF, or a derivative, are chemically bound to the protein of the liver as well as to that of other tissues (*44–46*), and preliminary studies suggest that this derivative may be involved in the induction of tumors.

A considerable amount of study has been directed toward determining the carcinogenic activities of metabolic derivatives and of structural analogs of these carcinogens. These studies have at least two major objectives—1) to determine whether the activity of a carcinogen can be ascribed to a metabolite, and thus carry the study to a compound closer to the primary carcinogen, and 2) to attempt to determine the sites on the molecule actually involved in the carcinogenic reaction.



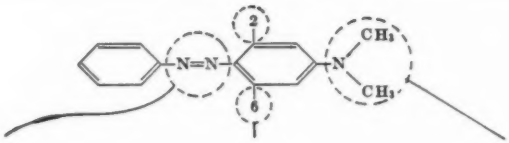
1) Deacetylation: $-NHCOCH_3 \rightarrow -NH_2$
2) Hydroxylation, primarily at position 7; lesser amounts at positions 1 and 3.
3) Binding to protein of liver and other tissues.

TEXT-FIGURE 2.—Reactions involved in the metabolism of 2-acetylaminofluorene.

The most pertinent data in this regard for DAB in rat liver have been reviewed recently (*2*) and are summarized in table 2. Those groups which appear to be essential to the carcinogenic activity of the molecule are enclosed in dotted circles. It

has been well established that at least one *N*-methyl group is required for high activity of the aminoazo dyes related to DAB. While a second methyl group is not essential and may be replaced, for example, by an ethyl group or a hydrogen atom, certain other substituents (hydroxyethyl and benzyl-methyl) apparently interfere with the metabolizability of the *N*-methyl group and thus make the dye inactive. The available evidence suggests that the requirement for at least one *N*-methyl group for activity is due to the involvement of this group in the reactions leading to the formation of the protein-bound derivative. The requirement for the azo linkage is not absolute since the analog 4-dimethylaminostilbene has considerable activity when account is taken of the lower doses administered, and 4-dimethylaminobiphenyl has weak activity toward the liver. On the other hand, neither the amines formed on complete reduction of the dye, the two possible Schiff bases, or the amide shown are carcinogenic when fed to rats.

TABLE 2.—*The importance of various groups in 4-dimethylaminoazobenzene for carcinogenic activity in the rat*

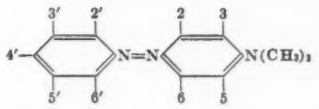


Active if	*Active if*	*Active if*
-CH=CH-	2-Fluoro-	-NHCH₃
		-N(CH₃)(CH₂CH₃)
Very weak if	*Inactive if*	*Inactive if*
Replaced by biphenyl linkage	2,6-Difluoro-	-NH₂
		-NHCHO
		-N(CH₃)CH₂CH₂OH)
		-N(CH₃)(CH₂Ø)
		-N[(CH₂)₁₋₄CH₃]₂

Inactive if
-NH₂+H₂N-
-CH=N-
-N=CH-
-CONH-

The apparent requirement that at least one of the positions *ortho* to the azo linkage on the diamine ring be unsubstituted is based on our studies of the fluorinated derivatives of DAB. As shown in table 3, substitution of the ring hydrogens with fluorine has generally yielded very active compounds (*5*, *47*). Thus, all the compounds in the first column are at least twice as active as DAB. These include three monofluoro derivatives (2,3′, and 4′) and several polyfluoro derivatives. Two dyes have activities about equal to that of DAB, and only the three dyes with substituents in both the 2 and 6 positions are inactive (*48*). This apparent requirement for one of the positions 2 or 6 to be open suggests several possibilities. For instance, the dye might undergo a rearrangement involving one of these positions to form the real carcinogen, or the 2-position might be involved in the binding to protein. Spectral data have been presented by Nye and Luck (*49*) which they interpret as indicating the presence of a 2-amino substituent in the polar dye isolated from hydrolyzed liver protein of rats fed 3′-methyl-DAB. On the other hand, an alteration in electronic or spacial configuration may have contributed to the inactivity of the 2,6-difluoro dyes.

TABLE 3.—*The effects of fluorine substitution on the carcinogenic activity of 4-dimethylaminoazobenzene*



	More Active	*As active*	*Inactive*
Monofluoro	4′-, 3′-, 2-	2′-, 3-	
Difluoro	2′,5′-, 3′,5′-, 2′,4′-, 3′,4′-		2,6-
Trifluoro	2′,4′,6′-		
Tetrafluoro			2,6,3′,5′
Pentafluoro			2,6,2′,4′,6′

The activities of these fluorinated dyes have also indicated that none of the positions on the prime ring are directly involved in the carcinogenic reaction. Thus, the high activities of 2′,4′,6′-trifluoro-DAB, in which the two *ortho* and the *para* positions are substituted, and of 3′,5′-difluoro-DAB, in which the two *meta* positions are substituted, essentially rule out reactions at these sites as being necessary for carcinogenesis. Studies on the polar dye isolated after alkaline hydrolysis of the protein-bound dye are in agreement with this idea. Thus, after reduction of the polar dye the prime ring can be obtained as the appropriate aniline derivative, so that the binding to protein must have taken place on the diamine ring or the *N*-dimethyl group (*50*).

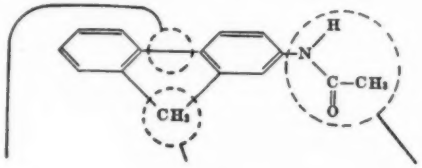
Data on the structural requirements for the hepatocarcinogenic activity of AAF in the rat are given in table 4 (*1, 6, 7, 9, 10, 42, 51–53*). Thus, a 2-amino or 2-acetylamino group appears necessary. The acetyl group is readily removed *in vivo*, and its importance in the carcinogenic process has not been ascertained. Morris and associates (*10*) have found the *N,N*-diacetyl derivative to be more active than AAF, and it seems possible that the major function of the acetyl groups is to protect the amine so that more is available at the critical sites. The nitro compound is considerably less active than AAF; possibly it is converted *in vivo* to the amino derivative. In contrast to the effects of *N*-methyl substituents in the dyes related to DAB, monomethylaminofluorene appears to be less active than AAF and dimethylaminofluorene relatively inactive for the liver.[3]

The methylene bridge in AAF has been of particular interest to us. Of the compounds tested in our laboratory with other groups in this position, only the fluorenone and fluorenol have had any activity toward the liver. These were both very weak, and it seems possible that they might be active through conversion in low yield either to 2-aminofluorene or to a carcinogenic derivative of 2-aminofluorene. As indicated in the table, several of the analogs inactive toward the liver are carcinogenic for other tissues of the rat. For instance, the thiophene analog has essentially the same activity as AAF for the mammary gland, ear duct, and small intestinal epithelium; the sulfoxide has definite though weaker activity toward these tissues. The phenanthrene and dihydrophenanthrene derivatives are both potent mammary carcinogens and 2-acetylaminophenanthrene is also leukemogenic. Even 4-acetylaminobiphenyl, which has no bridge in this position, is as active a mammary carcinogen as 2-acetylaminofluorene. Why the methylene bridge appears to be generally required for hepatic

[3] At these meetings Morris (*42*) reported that 2-dimethylaminofluorene was nearly as active as AAF in his tests.

carcinogenesis is not known and seems to be worthy of further study. 3-Acetylaminodiphenylmethane was inactive in our tests, so that a biphenyl linkage also appears to be necessary.

TABLE 4.—*The importance of various groups in 2-acetylaminofluorene for its hepatocarcinogenic activity in the rat*



Inactive if	*Weakly active if*	*Active if*
-H H-	-CO-	$-NH_2$
	-CHOH-	$-NHCH_3$
		$-NO_2$
	Inactive if	*Inactive if*
	-S-*	$-N(CH_3)_2$
	-SO*	-H
	-O-*	-Cl
	-CH=CH-*	
	$-CH_2CH_2-$*	
	-H H-*	
	$-SO_2-$	
	-NH-	

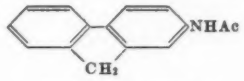
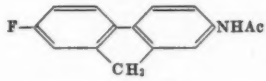
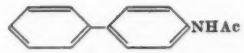
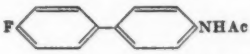
* Active at other sites.

As shown in table 5, the hepatocarcinogenic activity of AAF is enhanced by substitution with fluorine in the 7-position (*54*). High incidences of hepatic tumors were induced in both male and female rats fed low levels of the compound for 3 to 3½ months and then maintained on the basal diet for the duration of the experiment. These conditions were necessary because of the toxicity of the 7-fluoro-AAF. This enhancement may be due, at least in part, to the prevention of the hydroxylation of this compound at position 7, which in the case of AAF yields an essentially inactive compound (*55*). Data in this table also show that the substitution of fluorine in the comparable position of 4-acetylaminobiphenyl converts it to a weak hepatic carcinogen (*54*). Hepatic tumors were found in all the male rats fed 4′-fluoro-4-acetylaminobiphenyl which survived more than 6 months. Since 4-dimethylaminobiphenyl also has weak activity toward the liver (*6*), the methylene bridge cannot be considered as an absolute requirement, but it apparently facilitates some reaction necessary for hepatic carcinogenesis.

From this general survey of our knowledge what can we conclude about the mechanisms involved in carcinogenesis by these compounds? Because of the relatively small amount of information available for the other carcinogens, most of this discussion necessarily concerns DAB and its derivatives. Supporting evidence obtained with other compounds is included whenever possible.

An early suggestion was that DAB was carcinogenic by virtue of its reduction *in vivo* to *N,N*-dimethyl-*p*-phenylenediamine (*56*). This compound (or its oxidation products) is very toxic for certain enzyme systems, and it was suggested by Kensler and his associates that the induction of tumors might be a resultant of the poisoning of one or more enzymes in the liver. However, neither dimethyl-*p*-phenylenediamine, monomethyl-*p*-phenylenediamine, or *p*-phenylenediamine has induced either liver

TABLE 5.—*Hepatocarcinogenic activity of 7-fluoro-2-acetylaminofluorene and 4'-fluoro-4-acetylamir obiphenyl in the rat*

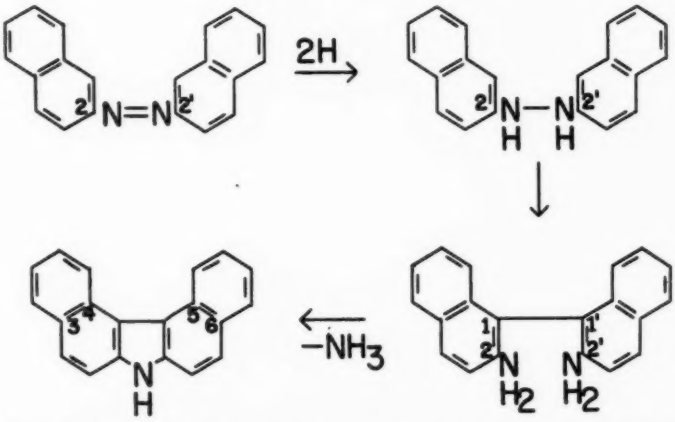
Compound	Percent in diet*	Months fed	Sex	Liver tumor incidence†		
				6 months	10 months	12 months
	0. 018	3	M	2/16	.....	
	. 028	3. 5	F	.....	0/13	
	. 020	3	M	12/16	.....	
	. 030	3. 5	F	.....	8/12	
	. 034	10	M	.....	.....	0/8
	. 037	10	M	.....	.....	5/8

*The composition of the grain diet is described in (*6*). Rats weighed approximately 200 gm. at start of experiment.

†Numerator indicates number of rats with hepatic tumors; denominator is number of rats in group.

tumors or liver damage when fed to rats at high levels for long periods (*2*). The other amines theoretically obtainable by reduction of the known azo metabolites of DAB have also proved inactive. Furthermore, high levels of dietary riboflavine strongly inhibit the carcinogenic action of DAB while favoring its reduction *in vivo* to the diamine and aniline (*2*, *57*). If the diamine were the carcinogenic agent, the reverse would be expected.

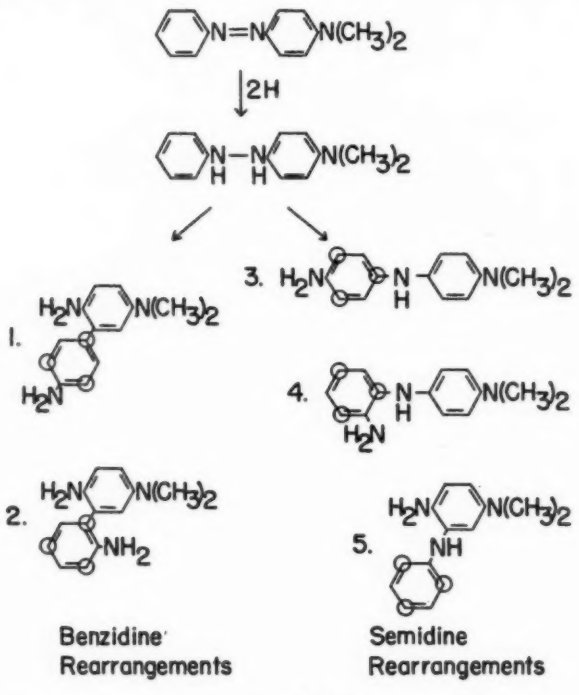
A second suggestion was that the aminoazo dyes were carcinogenic by virtue of their conversion to other products through intramolecular rearrangements. Cook and associates (*15*) working with 2,2'-azonaphthalene, which is carcinogenic for mouse liver, suggested that it might be active through *in vivo* conversion to 3,4,5,6-dibenzcarbazole which has also been demonstrated to induce liver tumors in mice. Thus, as shown in text-figure 3, this would involve reduction of the azonaphthalene to the



TEXT-FIGURE 3.—The possible rearrangement products of 2',2'-azonaphthalene in the mouse.

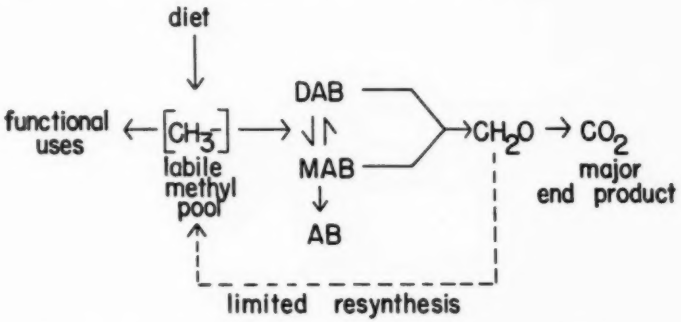
hydrazo derivative, followed by a benzidine rearrangement to the corresponding diaminodinaphthyl, and finally deaminative cyclization to the dibenzcarbazole. No evidence that this series of reactions takes place *in vivo* has been presented. In addition, the demonstration by Bonser *et al.* (*16*) that β-naphthylamine is carcinogenic for mouse liver suggests that the activity of 2,2′-azonaphthalene might be due to its reduction *in vivo* to two molecules of β-naphthylamine.

A similar suggestion for DAB was made by Elson and associates (*58*, *59*) on the basis of studies on metabolites of azobenzene in the urine of rats and on the toxicity of the benzidine rearrangement product of DAB, 2,4′-diamino-5-dimethylaminobiphenyl, for certain enzyme systems. From the data now available there seems to be little reason to believe that either benzidine or semidine rearrangements play a role in carcinogenesis by DAB (*2*). As shown in text-figure 4, two benzidine and three semidine rearrangements would be possible after reduction of DAB to the hydrazo derivative. Of the possible products the one suggested by Elson (No. 1) has been fed for 10 months at twice the molar level usually used for DAB; neither liver damage nor tumors were found. In addition, it and compounds 2, 3, and 4 are ruled out by the high activity of 2′,4′,6′-trifluoro-DAB. These considerations leave only compound 5 of this group as a candidate carcinogen. The inactivity of 2,6-difluoro-DAB is in line with this possibility. However, there is no evidence that compound 5 is formed *in vivo*. Moreover, we have now fed this compound to rats at a level equivalent to 0.06-percent DAB for 4 months; at this time there is no gross liver damage and no evidence of liver tumors.



TEXT-FIGURE 4.—The possible rearrangement products of 4-dimethylaminoazobenzene.

Since liver damage and hepatic tumors can be induced in rats and mice by a deficiency of methyl groups (*60, 61*), the possibility presents itself that the activity of hepatic carcinogens may be due to the induction of local methyl deficiencies. This deduction seems especially plausible in the case of DAB where the methyl groups of the dye are oxidatively removed *in vivo* (text-fig. 5). As shown by experiments with *N*-methyl-labeled DAB the methyl groups are eventually lost as expired CO_2, and additional methyl groups are drawn from the labile methyl pool for the remethylation of MAB to DAB. This cycle could thus act as a drain on the labile methyl pool of the liver. However, there is no evidence that this "leak" of methyl groups plays a role in carcinogenesis by DAB (*2*). Thus, in our experiments, administration of choline or betaine as sources of methyl groups simultaneously with DAB or MAB had no effect on the final tumor incidence. Likewise, administration of nicotinamide or guanidoacetic acid as methyl acceptors simultaneously with MAB was without effect on the tumor incidence. Yet, the effect of the methyl donors was apparent from the less severe cirrhosis that resulted and the dye-fed rats receiving the methyl acceptors had more severely cirrhotic livers. Kensler (*62*) has also attempted to relate tumor induction by the dyes to an induced methyl deficiency. However, he suggested that the methyl deficiency might be due to decreased levels of certain enzyme systems involved in methyl transfers as a result of ingestion of the dyes.

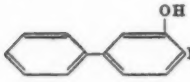


TEXT-FIGURE 5.—A possible mechanism for the production of a partial methyl deficiency in rat liver by 4-dimethylaminoazobenzene and its metabolites.

In the past few years certain of the British investigators (*16, 63, 64*) have championed the idea that the carcinogenicity of aromatic amines might be due to their conversion *in vivo* to *ortho* hydroxy amines. This suggestion appears to have real merit in the case of the induction of bladder tumors by β-naphthylamine. Thus, Bonser *et al.* (*65*) have correlated the excretion of 2-amino-1-naphthol in the urine by various species of animals with their susceptibilities to the induction of bladder tumors by feeding β-naphthylamine. She and her associates (*16*) have further shown that the implantation of paraffin pellets containing 2-amino-1-naphthol in the bladders of mice resulted in bladder tumors, whereas the similar implantation of paraffin pellets of β-naphthylamine did not. The recent report (*66*) that pellets of paraffin alone induced a high incidence of bladder tumors in rats, raises the question, however, of whether the paraffin also may have played a role in the induction of bladder tumors by the aminonaphthol in mice. Thus, it is possible that either the paraffin or the aminonaphthol was carcinogenic and that the other ingredient exerted cocarcinogenic activity. Also the demonstration of definite, though weak, carcinogenic activity for 3,3′-dihydroxybenzidine (*67*) can and has been used as evidence for the importance of *ortho* hydroxylation to the carcinogenicity of aromatic amines. At the present time, however, no convincing evidence has been presented to indicate that this metabolite is closer to the real carcinogen than is benzidine.

Walpole *et al.* (*63*) have similarly suggested that the carcinogenic activity of 4-aminobiphenyl and certain of its *C*-methyl derivatives (primarily for the mammary gland and intestine in his experiments) is due to their hydroxylation *ortho* to the amino group. They have assumed that metabolic data obtained with dogs were also applicable to the rat. Thus, in the dog, the presence of a methyl group *ortho* to an amino group favors hydroxylation in the remaining position *ortho* to the amino group. Accordingly, Walpole *et al.* have suggested that the greater carcinogenic activity of 2′,3-dimethyl-4-aminobiphenyl as compared to 4-aminobiphenyl for the rat is due to a higher degree of conversion of the former compound to the *ortho* hydroxy derivative. Whether or not these hydroxy derivatives are formed *in vivo* has not been reported. Experiments recently completed in our laboratory argue against this explanation. Thus, neither 3-hydroxy-4-aminobiphenyl nor its *N*-acetyl derivative was carcinogenic for male or female rats when fed in the diet for 10 months (table 6) (*54*). The only tumors found in these rats were fibroadenomas of the mammary gland; these occurred in as high an incidence in the control female rats fed only the basal grain diet. These rats were about 15 months of age at autopsy. The parent compound 4-acetylaminobiphenyl induced adenocarcinomas of the mammary gland in 10 of 13 rats by 6 months.

TABLE 6.—*Assay of 3-hydroxy-4-aminobiphenyl and its N-acetyl derivative for carcinogenic activity in the rat**

Compound	Percent in diet	Months fed	Tumor incidence, 12 months	
			Liver	Mammary gland
	0.034	10	0/13	12/13† (10/13 by 6 months)
	.037	10	0/8	1/8‡
	.029	10	0/7	3/7‡
None.....	.....	10	0/15	4/15‡

*Compounds were fed in a grain diet (*6*) to female rats, weighing approximately 200 grams at the start of the experiment.
†Adenocarcinomas.
‡Fibroadenomas.

Although we have no evidence that the 3-hydroxy derivatives of DAB or of its demethylated products are formed *in vivo*, 3-hydroxy-DAB has been synthesized and is now under test. After 5 months of feeding at a level of 0.064 percent to rats, neither liver damage nor tumors have been detected. Thus, if it should prove active at all, this derivative will be much weaker than the parent compound.

While it must be recognized that feeding a proposed metabolite does not insure that it will reach the sites at which it might be formed *in vivo*, we see no reason at the present time to believe that the activity of either the 4-aminobiphenyls or the aminoazo dyes is due to their *in vivo* conversion to *ortho* hydroxyamino derivatives. Since 2-aminofluorine is in essence a derivative of 4-aminobiphenyl, it appears unlikely that its activity will be explicable on this basis, but more data must be obtained in this regard.

For a number of years we have been investigating a protein-bound metabolite of DAB which occurs in the livers of rats fed the dye and which appears to be causally related to the induction of hepatic tumors (*2*).[4] The protein-bound derivative was originally recognized by its acid-base properties which are similar to those of DAB—*i.e.*, it turns reversibly pink in strong acid and yellow in neutral or basic media. Thus, if slices of perfused livers or the protein isolated from the livers are placed in trichloroacetic acid, those from rats fed DAB turn pink while those from normal rats are buff-colored. Early studies indicated that this dye was bound to the liver protein through covalent linkages. It could not be extracted from the protein by boiling solvents, by hot trichloroacetic acid which removes most of the nucleic acids, or by strong urea or detergent solutions. Dialysis of liver homogenates prior to precipitation of the protein also failed to remove the dye. The dye could be released from the protein only by prolonged enzymatic or alkaline hydrolysis. The major share of the dye thus liberated differs from that fed, in being very polar, possibly because of an attached amino acid or peptide fragment.

Investigations of the level of bound dye in various tissues and under various conditions led to the observation that tumor induction took place only when high levels of bound dye were formed in the first few weeks of dye-feeding. These correlations can be summarized briefly as follows:

1) Under most conditions tumors are found only in the liver of the rat, and this is the only tissue in which the protein-bound dyes can be detected.
2) Of the eight species tested, only two—the rat and the mouse—have proved susceptible to hepatic carcinogenesis by DAB. Similarly, these are the only two species in which the protein-bound dye can be detected. More bound dye is found in the liver of the rat, the more susceptible species, than in the liver of the mouse.
3) MAB, the only known carcinogenic metabolite of DAB, gives rise to the same level of bound dye as DAB, while the essentially noncarcinogenic metabolite 4-aminoazobenzene yields only a low level of bound dye. Little or no bound dye has been detected following the administration of the noncarcinogenic hydroxylated aminoazo dyes, some of which are known metabolites of these dyes.
4) When the extents of protein-binding of several of the ring-monomethyl derivatives of DAB are compared, it is found that the rapidity with which the maximum level of protein-bound dye occurs can be correlated directly with the carcinogenicity of the dye. With two exceptions, the levels of bound dye in the early weeks of dye-feeding are roughly proportional to the carcinogenicities of the dyes.
5) The levels of bound dye in the liver are lower when the dyes are fed in diets (such as those containing high levels of riboflavine or low levels of certain polycyclic hydrocarbons) which inhibit tumor induction by the dye.

Three situations are known in which high levels of bound dye are found in the liver during the first few weeks of dye-feeding, but in which the tumor incidence is low. Two of these are the feeding of the very weakly carcinogenic dyes 2-methyl-DAB and 3-methyl-MAB.[5] In these cases the high levels of bound dye can be explained in part, but probably not entirely, by a slower rate of degradation of the bound dyes formed from these dyes. This has been studied by determining the half-lives of the bound

[4] Recently in a talk at the 126th national meeting of the American Chemical Society in New York, Sept. 12–17, 1954, Dr. N. P. Buu-Hoi (*69*) presented the hypothesis that carcinogens may act by occupying "holes" in proteins normally occupied by physiologic compounds needed to control growth. He visualizes these complexes as akin to the clathrates in which molecules are trapped within a second large molecular framework. Buu-Hoi cited our work on the protein-bound aminoazo dyes as supporting his hypothesis. However, he has overlooked our published data (*1*) which demonstrate that the dyes are bound to the liver protein by covalent linkages. The same is true for the protein-bound derivatives of the polycyclic aromatic hydrocarbons (*70–74*).

[5] In our initial report (*68*) on the activity of 3-methyl-MAB, 1 of 11 rats fed 0.06 percent of the dye for 7 months developed a liver tumor by 9 months. In a more recent experiment, 2 of 15 rats fed 0.054 percent of the dye for 8 months had liver tumors at 10 months.

dyes after withdrawal of the dyes from the diets. The half-lives of the bound dyes from the 2- and 3-methyl dyes are about 4½ days, while the half-life for the bound dye from DAB is about 3 days. The third situation is that of hypophysectomy. As shown by Griffin and his associates (*75*) untreated hypophysectomized rats fed 3′-methyl-DAB are very resistant to tumor induction. Experiments in our laboratory have shown that these rats have at least as high levels of free and bound dyes in the liver as intact rats restricted to the same food consumption. The half-life of the bound dye in the livers of hypophysectomized rats is not significantly different from normal. Thus, the formation of bound dye may be a necessary, but not sufficient, requirement for the induction of liver tumors by the aminoazo dyes; other factors may become limiting in some situations.

In differential centrifugation experiments some bound dye has been found in each morphologic fraction of rat liver cells. However, over 50 percent of the bound dye in the liver is associated with the soluble proteins. Of this, at least 80 percent is attached to a slow-moving fraction (at *p*H 8.6) which accounts for no more than 15 percent of the soluble proteins. The bound dye is *not* found in the tumors produced by continuous feeding of the dyes. Furthermore, the electrophoretic component of the soluble proteins which binds the dye in the liver is greatly reduced in quantity in the tumor (*76*).

From these correlations it has seemed reasonable to suggest a tentative hypothesis of carcinogenesis by DAB and related compounds. According to this suggestion the administered dye is metabolized to a derivative (which we have pictured as the *N*-hydroxymethyl derivative of DAB or MAB) capable of combining with certain liver proteins. This would be the initial site of action of the dye on the liver. These liver proteins, or at least certain ones of them, are considered to play key roles in the response of the cell to its intrinsic growth controls (that is, by competitive reactions for various substrates or coenzymes) and to the extrinsic growth controls (such as hormones) exercised by the rest of the organism. If the binding of these proteins with the dye metabolite reduces or prevents the further synthesis of the proteins, eventually in subsequent generations cells might result with less and finally none of the proteins originally attacked by the dye. As a result of the loss of one or more key proteins controlling the rate of growth, these cells could respond to nutrition only by continued growth and would thus be tumor cells. The very low levels of the soluble proteins (which bind the dye in the liver) in the tumors is in harmony with this hypothesis of protein deletion.

Although the centrifugation and electrophoresis data show that there is some specificity in the proteins attacked, not all the proteins affected need necessarily play a role in the carcinogenic reaction. Too, it is possible that the soluble proteins which bind the dye may not be the key proteins and may serve only as indicators of a similar reaction in another part of the cell which is less amenable to study at the present.

Less extensive studies on certain other carcinogens indicate that these, too, are bound to the protein of the susceptible tissue, and some correlative evidence has been obtained for their importance in the induction of tumors. Thus, Taki and Miyaji (*77*) have presented evidence for the binding of low levels of derivatives of *o*-aminoazotoluene to the liver proteins of rats. Both Weisburger, Weisburger, and Morris (*45*, *46*) and we (*44*) have reported that, after the administration of AAF labeled with C^{14} in the 9 position, C^{14} is found tightly combined with the liver protein. No data have been presented concerning the identity of this metabolite. However, since only trace amounts of $C^{14}O_2$ are found in the respired air (*40*), it seems most likely that the C^{14} is acting as an indicator for a considerable share of the molecule. Recently we have determined the amount of protein-bound C^{14} in the livers of several species of animals given a single dose of AAF-C^{14} by stomach tube. To exclude adsorbed C^{14}-containing compounds as a source of radioactivity, protein samples were prepared both in the presence and absence of mercaptoethanol. This compound has been found effective by Levine and Tarver (*78*) in preventing the adsorption of ethionine on protein and in our laboratory in preventing the adsorption of C^{14}-containing aminoazo dyes or metabolites on various tissue proteins. The radioactivities of the protein samples

were not significantly affected by the mercaptoethanol, and thus appear to be a measure of chemically bound C^{14}.

As shown in table 7 the highest levels of bound C^{14} were found in the perfused livers of male and female rats. Rats, especially the males, are very susceptible to the induction of liver tumors by this compound. In our experiments the over-all incidence in female rats is low, but gross liver damage is evident by 5 or 6 months and most of the rats die soon thereafter with mammary tumors. An intermediate level of bound C^{14} was found in the livers of hamsters. In our laboratory, hamsters have developed severely fibrotic and cystic livers and a low incidence of liver tumors when fed 0.036 percent of AAF in a grain diet for 10 to 14 months. The next lowest level of bound C^{14} was found in mouse liver protein. Approximately 40 percent of the mice of the strain used will develop liver tumors after 10 months of feeding, but the level of carcinogen necessary was almost three times that fed to the animals of the other species in the tumor-induction experiments. Neither liver damage nor tumors have been found in either cotton rats or guinea pigs fed AAF for up to 18 months, and these animals had the lowest levels of protein-bound C^{14}.

TABLE 7.—*Relative amounts of protein-bound C^{14} in the livers of various species of animals after administration of 2-acetylaminofluorene-9-C^{14}* *

Species	Sex	Liver (perfused) counts/min./mg. of protein	Liver tumors	
			Percent	Months
Rat	M	92, 112	80–100	5–6
	F	97, 116	0–10 (liver damage)	6
Hamster	M	30, 32	10	14
Mouse	M	17, 19	40†	10
Cotton rat	M	12, 13	0	18
Guinea pig	M	9, 10	0	16

*Each animal received 22.7 mg. of 2-acetylaminofluorene-9-C^{14} (5,000,000 counts/min./mg.) per kg. of body weight in corn-oil solution (2.7 ml. per kg.) by stomach tube. Twenty-four hours later the animals were killed, their livers were perfused *in situ*, and the protein from liver homogenates was precipitated with trichloroacetic acid, washed with pH 5 acetate buffer and ethanol, and extracted in a Soxhlet apparatus for 48 hours with ethanol. The proteins were then dried and plated on aluminum disks for counting. We are indebted to Dr. C. Heidelberger for the 2-acetylaminofluorene-9-C^{14}.

†The mice were fed a purified diet containing 0.1 percent 2-acetylaminofluorene; all the other species were fed the grain diet (*6*) containing 0.036 percent.

Ethionine has been reported by Popper *et al.* (*19*) to induce severe liver damage and microscopic tumors in rats fed the compound as 0.2 percent of the diet for about 3 months. It is of interest that Levine and associates (*78*, *79*) have reported the incorporation of ethionine into the liver protein of rats. Here, the tumor induction may result from the production of an abnormal protein differing from its normal counterpart only by the subtle substitution of ethionine for methionine.

Similarly, experiments with 3,4-benzpyrene, utilizing either fluorescence or C^{14} as the indicator, and with radioactive 1,2,5,6-dibenzanthracene have shown that derivatives of these compounds are bound to proteins of the susceptible tissues (*70–74*). While a detailed account of these experiments is not pertinent in a conference devoted to hepatomas, they serve as further examples of the formation of protein-bound derivatives of carcinogens in susceptible tissues. They further strengthen the suggestion that the formation of such derivatives may be involved in the induction of tumors by many or all chemical carcinogens.

We would like to close with some general comments. It is true, of course, that the fundamental structure of the cancer cell which makes it relatively unaffected by the factors which govern the growth of normal cells has not been defined. However, whatever the alteration from normal is, it does appear to be both irreversible and heritable. From our present knowledge of cell composition and genetics, it appears

almost certain that such characteristics must be the result of changes in proteins or nucleic acids or both. These structures seem to be the only ones capable of existing in enough different forms to retain specific "information" within cells and to transmit it to subsequent cell generations. Surprise is occasionally expressed at the diversity of the chemicals and radiations which can induce neoplasms. But no one seems surprised at the great variety of compounds and radiations which can react with these complex structures. Hence, the wide variety of chemical and physical carcinogens is almost to be expected. While the data available indicate that proteins may be the site of attack in carcinogenesis by the aminoazo dyes and the hydrocarbons, it seems reasonable to expect that nucleic acids will be found to be the locus of action in other instances. Similarly, the firm combinations of certain carcinogens with these macromolecules are likely to be discovered first. Weak combinations and reactions between these macromolecules and carcinogens not involving binding seem equally possible but may be more difficult to detect. The really surprising things are the manifest stability of the organism in a variable and often inimical environment and the fact that the malignant change is not seen even more frequently than it is.

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Quantitative Histochemical Observations on Butter-Yellow Carcinogenesis[1, 2, 3]

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Two years ago we began a quantitative histochemical study of the well-known morphologic alterations—both neoplastic and non-neoplastic—which are found in the livers of male Harvard rats fed a semisynthetic diet (*1*) containing 0.06 percent *p*-dimethylaminoazobenzene (butter yellow). Our interest has centered around some phosphorus compounds and some of the enzymes associated with phosphorus metabolism because of the central position occupied by these compounds in energy transfer. We wish to present one of the results of this study.

Our methods are, in essence, histologically controlled microbiochemistry and are derived from those described by Linderstrøm-Lang and Mogensen (*2*). At varying intervals of time after being placed on the diet, animals were sacrificed by decapitation and the livers were quickly removed and frozen in isopentane chilled with liquid nitrogen. Serial sections 3 to 5 mm. in diameter and 20 microns thick were cut in a cryostat maintained at −18° C. Usually 5 serial sections were analyzed as a group. One section from each group was fixed and stained and served as a histologic control. The other sections were placed in siliconized micro test-tubes in which the chemical and enzymatic analyses were done. An attempt was made to obtain at least three sets of sections with a similar histologic picture from any given area of interest in the livers. The ribonucleic acid (RNA) of these sections was measured by a modification of the Schmidt-Thannhauser and Schneider procedures (*3*) which utilizes ultraviolet absorption. The *ortho*phosphates and easily hydrolyzable organic and pyrophosphates ($P_i + P_{18}$) were measured in that fraction of the tissue sections soluble in 0.3 N perchloric acid. The organic and pyrophosphates were converted to *ortho*phosphate by heating at 100° C. for 15 minutes in 1 *N* acid. All of these data were based on protein as a standard of reference. The protein has been measured in an adjacent comparable section, or in some instances of phosphate determination on the same section, by the method of Lowry *et al.* (*4*).

One of the questions we had asked ourselves concerned the possible relation between the acid-soluble phosphorus compounds and the phosphorus compounds of higher molecular weight in the changing morphology of the liver under the influence of the azo dye. It was found in all instances that the histologic control slides could be placed in one of 15 categories depending on the morphology of the sections. All the chemical data were distributed into these categories on the basis of the histology

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[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 12, 1954.

[2] This investigation was supported by a grant from the National Cancer Institute (C558), U. S. Public Health Service.

[3] This is publication #842 of the Cancer Commission of Harvard University.

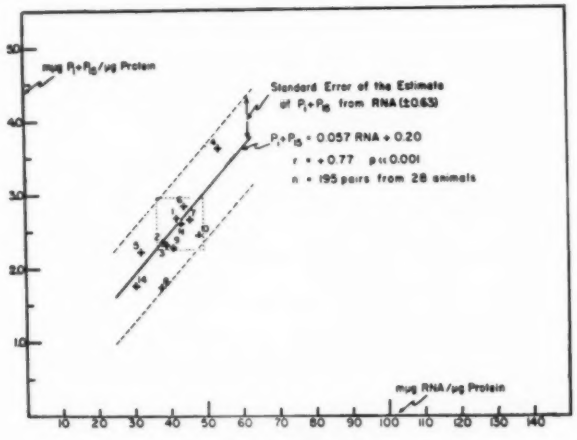
[4] Scholar in Cancer Research of the American Cancer Society. Associate in Medicine (Harvard University) at the Massachusetts General Hospital.

Associate in Pathology (Harvard University) at the Massachusetts General Hospital.

of the control slides. As we plotted a scattergram of the RNA *versus* the P_i+P_{15} it appeared that there was a trend.

A quantitative evaluation of the trend found in the scattergram is shown in text-figure 1. This is a summary of the data for all the normal rats studied and all the abnormal animals *except* the tumors. The crosses with adjacent numbers indicate the mean value for a given morphologic category. In the center is the mean for all the normals enclosed in a dotted rectangle which lies at three times the standard error of the means of the normals. The heavy line is the regression line calculated by the method of least squares for all the data except the tumors. The equation for this line is: $P_i+P_{15}=0.057$ RNA$+0.20$. The correlation coefficient is $+0.77$ and P is much less than 0.001. The dash lines parallel to the regression line are plus and minus one standard error of the estimate of the line.

From this analysis of the data, three facts appear: There is a well-defined proportionality in these livers between RNA and P_i+P_{15} concentrations based on protein. None of the nontumorous abnormals differs significantly from this proportionality. The mean value for only four of the abnormal categories differs significantly from the mean of the normals.

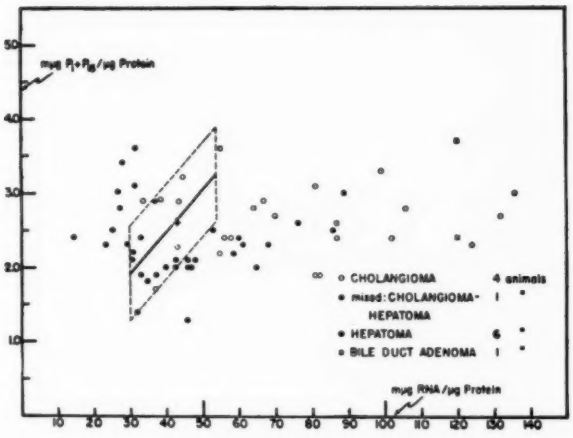

mug Pi+P15/ug Protein
Standard Error of the Estimate of Pi+P15 from RNA (±0.63)
Pi+P15 = 0.057 RNA + 0.20
r = +0.77 p << 0.001
n = 195 pairs from 28 animals
mug RNA/ug Protein
50
40
30
20
10
10 20 30 40 50 60 70 80 90 100 110 120 130 140

TEXT-FIGURE 1.

Since all the data from the normals and nontumorous abnormals fit the same regression line, no attempt will be made to give details of the morphologic definition of these categories for this presentation. The whole gamut of changes described in the literature is represented.

In text-figure 2 the data from the first figure have been enclosed in a parallelogram which contains all the means for the nontumorous categories. The regression line and its standard error remain as before. Superimposed on this is a scattergram of all the tumor data. Each point on the scattergram represents one pair of analyses; the types of tumor are noted in the legend. It is immediately apparent that the proportionality discussed above no longer exists. In tumors the variation in RNA is essentially independent of P_i+P_{15}.

We are aware that differences between tumors and nontumorous pathologic (pretumorous) alterations have been described by others only to have subsequent investigation reveal that embryonic and/or regenerating homologous tissue is similar to the tumors. With this in mind, we have analyzed the livers of 11 rats 48 hours after surgical partial hepatectomy. The data from these animals fit the regression line shown on these figures. We plan to study late embryonic liver and liver known to be meta-



TEXT-FIGURE 2.

bolically overactive. In addition, we are undertaking an exploration of the possible metabolic significance of the relation we have demonstrated.

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The Carcinogenicity of Ethyl Derivatives of *p*-Aminoazobenzene[1]

KANEMATSU SUGIURA, C. J. KENSLER, *and* M. L. CROSSLEY, *Sloan-Kettering Institute for Cancer Research, New York, N. Y.*

In 1935, Sasaki and Yoshida published the first description of liver cancer in rats due to the oral administration of *o*-aminoazotoluene. It took 300 days. In 1937, Dr. Kinosita produced liver cancer in a much shorter time (150 days) using *p*-dimethylaminoazobenzene. Since then many aminoazobenzene derivatives have been tested for carcinogenic activity in the rat, notably by Drs. Miller and Miller. It has been demonstrated that most changes of substituents on the parent molecule markedly affect carcinogenic activity. The present report deals with the carcinogenicity of ethyl derivatives of *p*-aminoazobenzene.

A 3-percent solution of *N,N*-dimethyl-*p*-aminoazobenzene and molar equivalent concentrations of other compounds were prepared in olive oil, which was mixed with brown rice (20 cc. per kg. of rice).

As may be seen from the data in table 1, the replacement of a methyl group by an ethyl group decreased or abolished the ability to produce liver tumors with one exception. The ethyl compound that showed high activity was *N*-methyl-4′-ethyl-*p*-aminoazobenzene (tumor incidence 100 percent). An ethyl group in the 2′-position of *N*-methyl-*p*-aminoazobenzene abolished activity, whereas the 2′-methyl derivative showed low activity (incidence 27 percent). A methyl group in the 3′-position showed the greatest activity. What effect an ethyl group in the 3′-position would have, I do not know at this time. Similarly, ethyl substituents on the amino group decreased or abolished carcinogenic activity: *N*-ethyl-*N*-methyl-*p*-aminoazobenzene (incidence 67 percent); *N,N*-diethyl-*p*-aminoazobenzene (incidence 0 percent).

The hydroxylation of *N,N*-dimethyl-*p*-aminoazobenzene in the 4′-position abolished carcinogenic activity. The hydroxylation of *N,N*-dimethyl-3′-methyl-*p*-aminoazobenzene in the 4′-position also abolished carcinogenic activity.

The reason for the high activity of the 4′-ethyl derivative is not apparent. One possible explanation for the low activity of the 4′-methyl compound and the high activity of the 4′-ethyl compound might be that the 4′-methyl group is readily oxidized and the 4′-ethyl is not. Oxidation of the 4′-methyl group would be expected to decrease or abolish the carcinogenic activity of the parent molecule, as methyl orange, as well as the 4′-OH derivative, is inactive. However, Dr. Kensler's *in vitro* experiments with 4′-ethyl, 4′-methyl, and *N,N*-dimethyl-*p*-aminoazobenzene, using liver slices, indicate that the marked difference in carcinogenic potency between the 4′-ethyl and 4′-methyl compounds is not due to rapid oxidation of the 4′-methyl group.

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 12, 1954.

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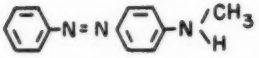
TABLE 1

Compound	Percent Tumor Incidence
	93
	93
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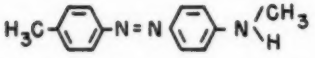
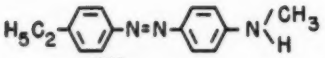
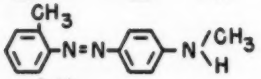
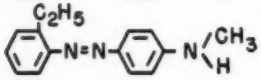
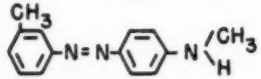
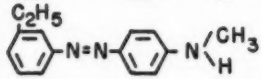
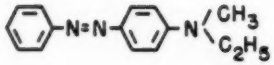
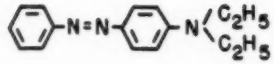
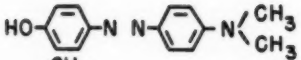
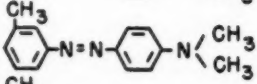
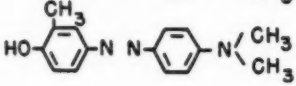
	20
	100
	27
	0
	100
	?
	67
	0
	0
	100
	0

Hepatic Lesions Caused by Protracted Ethionine Feeding[1,2]

HANS POPPER, M.D. *and* CLARA BRUCE, *Hektoen Institute for Medical Research of Cook County Hospital and the Departments of Pathology of Cook County Hospital and Northwestern University School of Medicine, Chicago, Ill.*

Several years ago Dr. E. Farber, who had worked with Greenberg at the University of California, studied in our laboratory the morphologic effects of ethionine, which is an analogue of methionine (an ethyl group replacing the methyl group) and apparently also its biologic antagonist. Administration of single doses leads to fatty liver in female rats, but not in males, and pancreatic changes in animals of both sexes. Studies concerning various morphologic and functional aspects of the ethionine intoxication (*1–5*) included prolonged feeding of a synthetic diet containing 0.5-percent ethionine and approximately 0.4-percent methionine in its protein. This diet resulted in pancreatic degeneration and atrophy as well as in a diffuse hepatocellular degeneration and regeneration, bile-duct proliferation, interstitial inflammatory infiltration and fibrosis. The regenerating liver cells were often bizarre in shape, multinucleated and revealed large nucleoli. As a rule, rats do not survive on this diet for more than 60 days. Some of them, however, did survive; others were alternated on the ethionine and a stock diet (Checkers) or were kept on an 0.2-percent ethionine diet. In such rats, as stated a year ago (*6*), small nodules developed composed either of bizarre proliferations of the bile ducts associated with periductular fibrosis (cholangiofibrosis) or of liver cells arranged in plates several cells thick and markedly differing from the surrounding parenchyma.

These experiments have been extended in the past year with variation of the concentration of the ethionine content and the spacing of the diet in a so far unsuccessful attempt to produce unquestionable hepatic carcinoma. To date, a total of 114 rats have been on the 0.5-percent ethionine diet for periods from between 80 to 729 days, in experiments in which the maximal period was 208 days. In addition, 20 rats have been on an 0.2 percent ethionine diet from 97 to 439 days.

At this time three examples are demonstrated. In a rat that has been on an alternating 0.5 percent ethionine and Checker diet for 14½ months, a fine nodular cirrhotic liver was noted. In addition, several larger nodules up to 0.5 mm. in diameter protruded from the surface (fig. 1). Histologically, the liver was composed of hyperplastic nodules without recognizable portal and central canals in which inflammatory exudate accumulated occasionally around degenerating cells. Between the nodules extensive inflammation, bile-duct proliferation and cholangiofibrotic foci were noted (fig. 2). The large nodes protruding from the surface consisted of liver cells which were much larger than those in the rest of the liver; the nuclei were large and revealed large nucleoli and they were arranged in plates several cells thick to produce a blastema-like picture (fig. 3). In silver impregnations the reticulum framework in these large nodules was sparse and was much less impregnated than in the rest of the liver. Mor-

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 12, 1954

[2] Supported by U. S. Public Health Service grants A-334 and C-2030.

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phologic inspection did not permit a clear-cut decision as to whether they represent hyperplastic or neoplastic nodules, but vascular invasion or metastases were not found and malignancy can be excluded. The spleen was excessively large and the serum *gamma*-globulin level markedly elevated.

In another case with a similar experimental history, the picture of the liver was grossly almost the same (fig. 5). However, the protruding nodules (which were up to 0.8 cm. in diameter and grayish white on the cut surface) consisted of cholangiofibrotic tissue (fig. 4) in which a large number of bizarre-shaped bile-duct proliferates filled with mucus were noted (fig. 6). They resembled lesions previously recorded (7): rats first receiving an alternating ethionine and Checker diet were subsequently kept for one year on a Checker diet. In large nodules consisting of sclerosing connective tissue actively proliferating bile ducts persisted.

A third rat was first kept on alternating 0.5-percent ethionine and Checkers diet for 134 days and subsequently on Checkers for 14 months, but was then returned to the alternating diet for another 4 months—accounting for a total experimental period of 2 years. After the animal was sacrificed, the large cirrhotic liver contained several large nodules; the largest, centrally necrotic, was 1.8 cm. in diameter (fig. 7). It consisted histologically of irregularly proliferating adenomatous structures (fig. 8). The epithelial lining showed marked hyperplasia in places, with piling up of cells and with many mitoses. In other nodules the epithelial lining was missing. The dilated lumen contained mucus which in places appeared free in the stroma (figs. 9 and 10). The lesion was unclearly delimited from the surrounding liver tissue and groups of liver cells were found throughout. Despite some similarity to a mucin-producing adenocarcinoma, neither venous invasion nor metastases could be demonstrated and the malignant character was not proved.

The figures presented reveal similarity to the early stages of the lesion (shown at this meeting by Drs. W. D. Salmon, D. H. Copeland, and M. J. Burns, of Alabama) which develops in long-standing choline deficiency and which precedes the development of unquestionable carcinoma. When observing these stages the Alabama workers wondered whether they represented a malignant lesion. The development of autonomous behavior of the hyperplastic cholangiofibrotic and hepatocellular nodules in a cirrhotic liver following prolonged ethionine administration is indicated by their persistence, even after discontinuation of the ethionine diet, and by the lack of the morphologic signs of the injury which the remaining parenchyma shows. This might suggest tentatively a neoplastic character of the lesion. However, the crucial question of malignancy is far from being proved. Nevertheless, the pictures obtained encourage a continued attempt to produce unquestionable malignancy, especially in view of the experiences of the Alabama workers.

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PLATE 134

Liver of rat that had been alternating on an 0.5-percent ethionine diet and on normal diet for 409 days (in toto *208 days on ethionine diet*).

FIGURE 1.—Large nodule protruding over the surface of a cirrhotic liver. × 4

FIGURE 2.—Regenerative nodules of various sizes embedded in dense connective tissue containing inflammatory exudate, proliferated bile ducts and a few scattered liver cells. The *left lower half* represents the protruding nodule composed of large liver cells arranged in plates several cells thick and traversed by a few connective trabeculae. × 80

FIGURE 3.—Close-up of *lower left half* of figure 2 showing large liver cells (with large nuclei and nucleoli) arranged in plates several cells thick without intervening stroma. × 295

PLATE 135

Liver of rat that had been alternating on an 0.5-percent ethionine diet and on normal diet for 412 days (in toto *208 days on ethionine diet*).

FIGURE 4.—Nodule consisting of dense connective tissue with isolated, hardly recognizable strands of proliferated bile ducts. × 4

FIGURE 5.—Gross appearance of cirrhotic liver with multiple white protruding nodules.

FIGURE 6.—Close-up border of fibrotic nodule revealing irregularly arranged regenerating liver cells surrounded by connective-tissue trabeculae with proliferated bile duct and inflammatory exudate. In the *upper right corner*, irregularly arranged, proliferated bile ducts embedded in dense connective tissue are noted; cholangiofibrosis. × 80

PLATE 136

Liver of rat that had been first alternating on an 0.5-percent ethionine diet and on normal diet for 134 days, then on normal diet for 529 days and subsequently again on the alternating diet for 166 days.

FIGURE 7.—Cut surface of liver revealing large nodule, irregularly limited and centrally necrotic.

FIGURE 8.—Border zone of nodule revealing cholangiofibrosis under the capsule and in the central portion, irregularly arranged glandular structures in a fibrosing stroma. × 60

FIGURE 9.—Mucinous material in the lumen of the glandular structures. The epithelial lining is missing in many places and the mucinous material lies free in the densely infiltrated connective tissue. × 120

FIGURE 10.—Irregular piling up of epithelium and mitoses in the lining of the glandular structures. × 330

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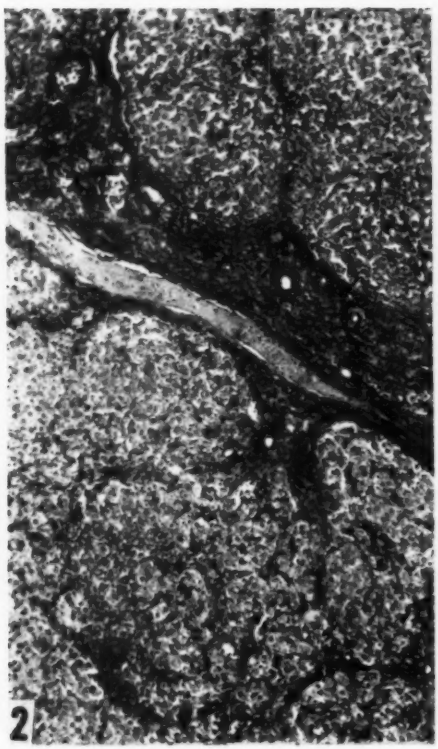

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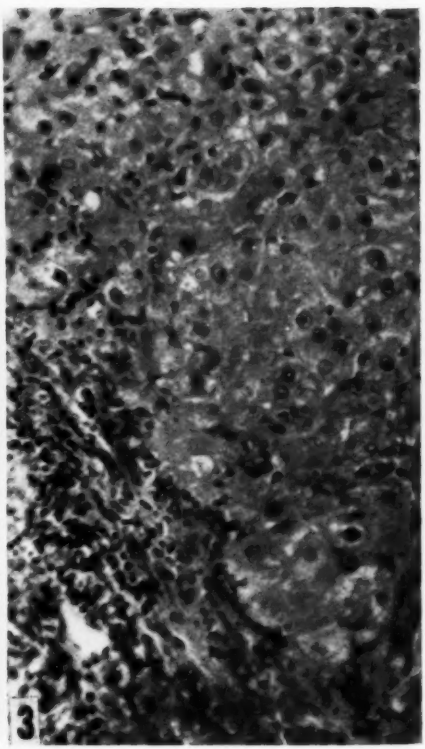

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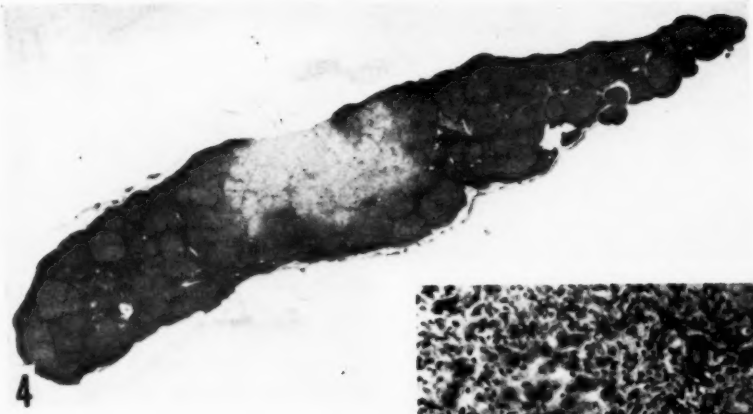
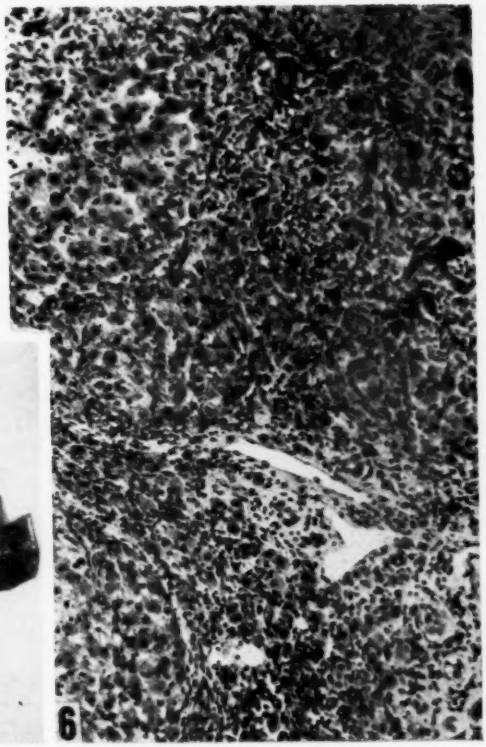
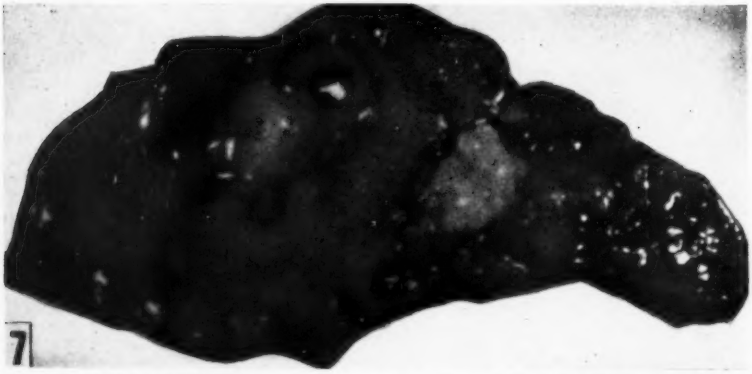
Popper and Bruce



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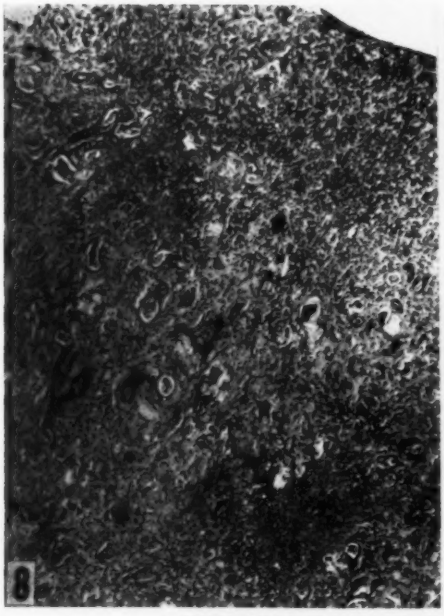
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The *In Vivo* Effect on Liver Catalase by a Tumor[1]

JESSE P. GREENSTEIN, *Laboratory of Biochemistry, National Cancer Institute,*[2] *Bethesda, Md.*

Biochemical studies on tumors beginning with the classic observations by Otto Warburg have strongly indicated that these tissues are characterized by the possession of a relatively uniform metabolic pattern which is independent of the etiology and histogenesis of the tumors. It is possible therefore to describe tumors in general, as hepatic and renal tissues may be described, in terms of limited and ascertainable properties. It must be emphasized, however, that it is the metabolic pattern which distinguishes these diverse tissues, and not necessarily the individual enzyme systems (comprising this pattern) which to a greater or lesser degree they may possess in common. Because of the overlapping metabolic characteristics which tumors share with normal tissues, a clear-cut chemical distinction between them is not always possible, and their differences can as yet be described only in quantitative and not qualitative terms.

The complete metabolic machinery of tumors has not so far been revealed, but it may be assumed on the basis of evidence now available, that in certain characteristics these tissues will surpass normal tissues and in others fall far below them. If the former are at all excessive, it is possible that they may be reflected in the behavior of the tumor-bearing host, and constitute one or more distinguishing characteristics of an individual in such a condition. The emergence of such characteristics, and their assessment in quantitative terms, would in turn support an essentially indirect approach to the study of extremely active metabolic systems in tumors—systems powerful enough to exert an effect or effects against a living and resistant background.

Experimental Observations

It was with these thoughts in mind, that a series of studies[3] was undertaken in the writer's laboratory beginning in 1942 on the levels of individual enzyme systems in various tissues of experimental animals bearing tumors at sites removed from the tissues studied. Of the large number of systems studied, only the liver catalase appeared to be unusually affected. This enzyme was considerably depressed in its level from the level characteristic of normal liver. The findings of these earlier studies may be summarized as follows: *a*) the liver catalase system was appreciably diminished only when the tumor reached a size about 5 percent of that of the total body weight of the host; *b*) the depression in this hepatic system was progressive with growth of the tumor; *c*) the hepatic system was restored to the normal level by 1 to 3 days after complete extirpation of the tumor; *d*) although the kidney and the erythrocyte contained

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 12, 1954.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] For references to these studies, see Greenstein, J. P.: Biochemistry of Cancer, 2d ed. New York, Academic Press, 1954.

considerable catalase, the level in the former was relatively little affected and the level in the latter not at all affected by the presence of a growing tumor elsewhere in the animal; and *e*) the liver catalase level in the pregnant animal, or one in which a mash of embryonic tissue was growing rapidly was not altered from the normal. At the risk of repetition, it is important to emphasize the fact that only the liver catalase of all the hepatic systems studied was markedly affected by the presence of a distant tumor—a generalized condition such as starvation or infection would be expected to affect most, if not all, hepatic systems. It seemed probable therefore that a specific biochemical lesion was being induced in the liver by the presence of a distant tumor.

This problem was further illuminated by the work of Nakahara in Tokyo, who isolated a crude fraction from tumors which upon injection into normal mice produced a lowering of the liver catalase in such mice; similar fractions from normal tissues apparently did not possess this effect. A more precise method of fractionation of tumors to isolate this active material was undertaken by Greenfield and Meister in the writer's laboratory. These investigators found that *a*) this material would on injection lower the liver catalase of normal animals, *b*) by 3 days after the injected dose the liver catalase was at its normal level, *c*) other hepatic enzymes were undisturbed by an injection sufficient to lower the hepatic catalase by one half, and *d*) similar fractions of normal tissues were obtained by identical methods of isolation to a much smaller extent than from tumors, but they produced a definite but slight depression of the liver catalase of animals in which they were injected. An inhibition of crystalline catalase and other porphyrin enzymes *in vitro* was demonstrated by Hargreaves and Deutsch, who employed an extract of tumors (*kochsaft*); enzymes other than those with a porphyrin prosthetic group were unaffected by the treatment. Boiled extracts of certain normal tissues, and to a greater extent, boiled extracts of such tissues from tumor-bearing animals, possessed the ability to inhibit the activity of crystalline catalase *in vitro*. It thus became apparent that the catalase-inhibiting factor shown to be a characteristic of tumors was not absent from many normal tissues but was present only in much lesser degree than in tumors.

In the meantime, a notable series of studies by Adams in London, by Lucké in Philadelphia, and by Begg in Western Ontario further demonstrated the progressive diminution in the hepatic catalase of tumor-bearing animals with growth of the tumor. The former investigator employed homogenized Sarcoma 37 in mice and revealed the following consecutive phenomena: *a*) an initial drop in the hepatic catalase shortly after inoculation of the tumor homogenate, due probably to a release of a toxic material (Nakahara fraction?) into the host, *b*) a restoration to the normal level of liver catalase at the fourth day, due to exhaustion of the supply of toxin with concomitant restorative powers of the host, and *c*) beyond the fourth day, when the tumor began to be actually palpable, and thereafter during the course of its progressive growth, the familiar progressive diminution in the liver catalase level of its host. With still finer homogenization of the tumor, the initial drop in liver catalase actually reached its lowest level at 1 day after inoculation, the normal restoration was advanced to 7 days, and the delay in tumor growth caused the lowering of the liver catalase levels to proceed only after the tenth day. Similar preparations of normal and embryonic tissues, similarly administered, produced no effect on the liver catalase of the animals. The studies by Lucké showed that the liver catalase level of the non-tumor-bearing partner in parabiotic union with a tumor-bearing animal was considerably depressed below the normal level, indicating that a humoral factor was involved. Lucké also developed a precise quantitative relationship between the decrease in liver catalase activity and the increase in the population of tumor cells in mice bearing the Ehrlich ascites tumor, a feat possible only with a tumor whose individual cells can be so exactly counted. The interesting observation that the liver catalase effect was not restricted to mammals but would also occur in frogs bearing a renal carcinoma was also made by Lucké. Begg disposed of the possibility that the liver catalase decrease might be related to a reduced food intake with consequent starvation of vital tissues by demonstrating such

a decrease in tumor-bearing animals which were force-fed to maintain their carcass weight.

The most recent studies in this field of cancer research have been conducted by Price and Greenfield in the writer's laboratory. These have involved the actual physical isolation of catalase from preparations of normal liver and of the livers of rats bearing the Novikoff tumor. These livers were morphologically and microscopically indistinguishable, and were of the same weight. The catalase fractions isolated by adsorption procedures on calcium phosphate gels were further fractionated into several chromatographic peaks on calcium phosphate-cellulose columns. This study revealed a considerable quantitative difference but no apparent qualitative difference in the catalase isolated from the livers of normal and of tumor-bearing animals, that in the latter being markedly diminished both in terms of the Soret band and in activity toward peroxide.

Conclusions

It is apparent that the presence of a growing tumor at a site removed from the liver produces a progressive diminution in the catalase level of the liver, which can be related both to amount and activity of the catalase in the liver. This effect appears to be specific for enzymes with a porphyrin group, and is reversible on removal of the tumor. The effect appears to be due to a heat-stable entity within the tumor which is humorally distributed. Why this entity does not affect the catalase of the kidney and of the erythrocyte to the extent that it does the catalase of the liver is at present inexplicable. This active material is apparently produced to a much smaller extent in normal tissues, and the difference in this function, like that of many other functions found in tumors and in normal tissues is more of degree than of kind. Like glycolysis which is relatively highly active in tumors as compared with normal tissues, the capacity of tumors to produce the hepatic catalase toxin is much higher than in normal tissues—higher to such an extent that it spills over into the blood stream of the host. That which is a toxin in a tumor due to abnormal production may be only a normal regulator of enzyme levels in a normal tissue, and illustrates a phase of the biochemical as well as morphologic disequilibrium which may ensue in a host bearing so foreign a tissue as a growing tumor. Whether this abnormal toxin production is related to the abnormal growth properties of the tumor and consequently to its intrinsic metabolism, or whether it is merely an adventitious retention of a normal property to a bizarre degree, can be ascertained only by further experiment.

Enzymatic Properties of Rat Liver Tumor Mitochondria [1,2]

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The tremendous amount of work done by investigators of various disciplines on experimental liver cancer has revealed the complexity of tagging a biochemical defect as a specific cellular damage leading to neoplasia. It is even a problem of much concern today, to recognize a biochemical alteration which is characteristic of tumor cells.

The problem is complex because one has to cope with many incidentals which limit the interpretation of biochemical data obtained either with tissue extracts, slices, or homogenates. For instance, one of these incidentals is the important fluctuation of the cellular population in liver tumors as well as in liver during carcinogenesis (*1*).

In our effort to bridge straight biochemistry, and especially enzymology, with quantitative cytology, advantage has been taken of the useful "macrodissection" technique developed by Hogeboom, Schneider, and Pallade (*2*), to prepare different cellular fractions by differential centrifugation. Of these liver fractions, the author in collaboration with Dr. G. de Lamirande[3] has been especially interested in the mitochondria.

Results obtained in the course of these studies on the behavior of mitochondria have suggested, for instance, that mitochondria isolated from primary rat liver tumor possessed the same level of activity of acid phosphatase (acid PO_4ase) (*3*) and deoxyribonucleodepolymerase (DNAase) (*4*) as normal liver mitochondria. On the other hand, the soluble protein composition of tumor mitochondria was shown to be seriously altered (*5*) as well as the nucleotide composition of ribonucleic acid (RNAases) (*6*).

Recently the acid and alkaline phosphatases and acid and alkaline RNAases were studied systematically in regenerating and "precancerous" livers and in primary liver tumors. It is our purpose to discuss here some data obtained with the mitochondrial fraction. The latter fraction was isolated in 0.25 M sucrose as described by Schneider (*7*). Methods and enzymatic-assay procedures are not given here since they were covered elsewhere (*8–10*).

Table 1 shows in detail results on acid and alkaline RNAases and acid and alkaline PO_4ases activity of mitochondria isolated from adult Wistar rats as compared to those of 4-dimethylaminoazobenzene (DAB) primary tumors. These tumors were induced by feeding a low protein semisynthetic diet as described previously (*11*).

Table 1 (1st and 2nd column) shows that the activity of the RNAases remained the same when calculated on a fresh weight basis; whereas the specific activity (per μg. nitrogen) as well as the activity per average mitochondrion was increased significantly. Nevertheless the average tumor cell possessed the same mitochondrial activity as the normal average liver cell as shown by results calculated per nucleus (actually counted in the original homogenate). Thus, with respect to RNAase activity these facts suggest that either the activity was increased in all large granules or preferentially in some of them. It remains to be seen also whether or not these

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 12, 1954.

[2] This study was aided by grants from the National Cancer Institute of Canada to Dr. A. Cantero, Director of the Research Laboratories.

[3] Fellow of the Damon Runyon Memorial Fund for Cancer Research, Inc.

TABLE 1.—*Enzymatic properties* of mitochondria isolated from rat liver and primary liver tumor induced by 4-dimethylaminoazobenzene*

Enzymes†	RNAases				PO_4ases					
	Acid		Alkaline		Acid		Alkaline			
							With Mg^{++}		Without Mg^{++}	
Tissues	Liver	Tumor‡	Liver	Tumor	Liver	Tumor	Liver	Tumor	Liver	Tumor
Activity per fresh weight of tissue§	185 ±43	167 ±42	360 ±97	301 ±65	648 ±95	234 ±40	34 ±7	213 ±101	25 ±10	183 ±70
Percentage of the original homogenate	55 ±5	33 ±4	53 ±2	35 ±8	58 ±4	31 ±3	8 ±1	17 ±1	16 ±3	17 ±2
Specific activity	22 ±5	43 ±18	43 ±11	77 ±35	767 ±77	510 ±238	40 ±8	610 ±540	31 ±5	510 ±331
Relative specific activity	(1.8)	(1.9)	(1.7)	(2.0)	(2.0)	(1.4)	(0.3)	(1.0)	(0.7)	(1.0)
Activity per cell‖ $\times 10^6$	1.2 ±0.3	1.0 ±0.5	2.4 ±0.6	1.8 ±0.8	50 ±16	18 ±7	25 ±2	14 ±12	1.8 ±0.5	11 ±5
Activity per mitochondrion ¶ $\times 10^{10}$	5.1 ±1.4	12.7 ±6.4	9.3 ±2.2	22.8 ±11.1	217 ±33	182 ±47	11 ±3	171 ±132	8 ±2	137 ±79

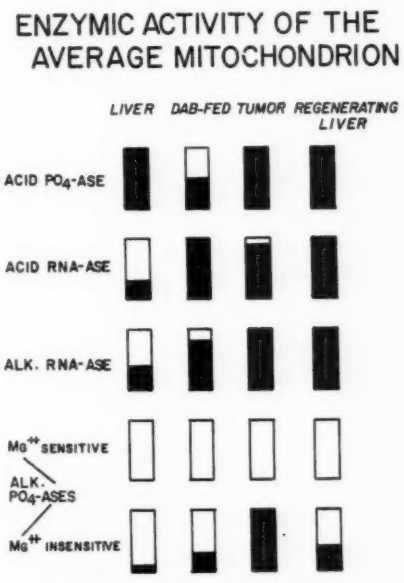
*Mean of 4 to 6 determinations with standard deviation.
†Assayed as described in (*8, 10*)
‡1–1½ cm. in diameter—induced as described in (*11*).
§Activity calculated as in previous papers (*8, 10*).
‖Per average nucleus.
¶Per average mitochondrion as previously described (*9*).

RNAase-rich granules are localized in cell types found in tumor but to a small extent in healthy liver and also whether all large granules in normal liver and tumor cells contain the same activity. (A transplantable rat hepatoma is now being studied in which the cellular population is mostly composed of neoplastic parenchymal cells.)

With respect to acid PO_4ase, lower activity was measured per fresh weight of tumor tissue as compared to liver. However, the specific activity as well as the activity per average mitochondrion of tumor was similar to the normal liver. This fact indicates that the mitochondrial activity per fresh weight was less in these tumors because the number of mitochondria was lower than in liver cells (*11*). With respect to this enzyme, the average tumor mitochondrion was not altered.

Table 1 (last column) shows, however, that tumor mitochondria possessed alkaline PO_4ase activity, whereas normal liver mitochondria did not (*8*). Also, as illustrated in table 1, Mg^{++} ions did not significantly activate alkaline PO_4ase activity. This fact suggests that only the Mg^{++} insensitive (*8*) alkaline PO_4ase is increased in these neoplastic tissues. It is of interest to note that the range of activity in tumor mitochondria was very large as shown by the great standard deviation. Whether or not alkaline PO_4ase observed in liver primary tumor mitochondria corresponds to this enzyme being linked to large granules remains to be proved. However, according to the relative specific activity (ratio of mitochondrial to original homogenate specific activity) which was 1.0 (table 1, last column) alkaline PO_4ase would appear to be present in the granules found in this fraction of the tumor homogenate.

Because space is limited, additional data on regenerating liver and "precancerous" liver have been summarized in text-figure 1 and combined with those described above.



TEXT-FIGURE 1.—The blocks represent relative percentage of enzymic activity of the average mitochondrion isolated from various rat liver tissues. The highest enzymic activity in any of the conditions studied was taken as 100-percent activity. The DAB-fed rat livers were from rats fed the azo dye continuously for 40 and 80 days and were compared to adequate controls (basal diet alone). Regenerating livers correspond to 18 hours and 3 days post-66 percent hepatectomy and were compared to sham controls. For liver and tumor tissues, see text.

Text-figure 1 illustrates for instance that the average mitochondrion of tumor, and regenerating and DAB-fed livers possessed the same level of activity of acid and alkaline RNAases, but that such activities were much higher than in normal liver. It was found also that only in tumor was the Mg^{++} insensitive alkaline PO_4ase activity much greater than in liver, though it was increased in all the other conditions studied. Only in the case of acid PO_4ase did tumor mitochondria contain the same activity as in liver. However, surprisingly enough, in DAB-fed animals, this enzyme activity was about half that of normal tumor or regenerating average mitochondrion. These results expressed per average mitochondrion actually counted were essentially the same as on a per nitrogen basis.

This brief presentation of a few data on the enzymatic behavior of primary liver tumor mitochondria as compared with liver under various pathologic and physiologic conditions, does not permit any sound evaluation at this time. Nevertheless, the data suggest that the large granules isolated from liver tumor cells are surely not biochemically equivalent to those isolated from liver. How specific these changes are in relation to the neoplastic state remains uncertain.

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Rhodanese Activity of Resting, Regenerating, and Neoplastic Liver Tissue of the Rat [1,2]

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The enzyme rhodanese, discovered by Lang (*1*) in 1933 and crystallized by Sörbo (*2*) in 1953, catalyzes the conversion of hydrocyanic acid and thiosulfate into thiocyanate and bisulfite. The reaction offers a possible cellular mechanism for the detoxification of cyanide, although its biological significance is not completely understood at the present time.

In 1946, Mendel, Rudney, and Bowman (*3*) pointed out that there were only traces of rhodanese in transplantable sarcomas and certain normal rat tissues which, like tumors, displayed high aerobic glycolysis *in vitro*. The authors interpreted this finding to mean that the high aerobic glycolysis of normal and neoplastic rhodanese-deficient tissues was due to the inhibition of the Pasteur reaction by small amounts of cyanide formed in the intermediary metabolism of the cell.

We had questioned the validity of this interpretation when we found (*4*, *5*) that various normal tissues known to show a pronounced Pasteur effect were as low in rhodanese as those with high aerobic glycolysis. It was also pointed out by us that the tumors studied by Mendel and his group were derived from rhodanese-poor types of normal cells.

After this study was completed we had the opportunity of assaying a few experimental hepatomas of the rat. Rat liver contains 10 to 20 times more rhodanese than all other tissues of the rat, with the exception of kidney cortex. It is also considerably more active than liver tissue from any other mammalian species thus far studied. Rhodanese activity thus represents a characteristic biochemical property of rat liver. The fate of the enzyme during the malignant transformation of the cells appears of particular interest. Moreover, comparative information is available on the changes in hepatic rhodanese levels during protein depletion (*6*), aminoazo-dye feeding, developmental growth (*7*) and regeneration (*8*), and on the intracellular distribution of the enzyme (*9*, *10*).

Rats with primary-induced dimethylaminoazobenzene tumors were obtained through the courtesy of Dr. Julius White, of the National Cancer Institute in Bethesda. The tumors to be studied were carefully freed of necrotic areas and extraneous material. Samples were then taken for rhodanese assay (*6*), moisture determination, and histologic examination. Dr. F. H. Burgoyne, of the National Cancer Institute, kindly interpreted for us microscopic sections from the specimens.

From table 1 it is seen that protein depletion decreased the rhodanese activity of rat liver and that regenerating tissue was less active than resting tissue. The values for the malignant hepatomas, though distinctly below the lowest normal value, were high in comparison with those for other rat tissues since only kidney cortex assayed

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above 100 units. Lower values were found in the anaplastic liver cancer containing areas of bile-duct proliferation and in the probably benign or hyperplastic fibrous tumor with extensive bile-duct proliferation. The latter structure is included in the series, as the low activity suggests that the biliary component of liver is poor in rhodanese.

TABLE 1.—*Rhodanese activity of normal and neoplastic liver tissue*

Tissue	Type of preparation*	Rhodanese units†
Liver	Protein-fed, resting	534
	Protein-fed, 2 days' regeneration	370
	Protein-depleted, resting	255
	Protein-depleted, 2 days' regeneration	222
Tumors	Malignant hepatoma	165
	Malignant hepatoma	118
	Anaplastic tumor+ductal proliferation	56
	Fibrosis+ductal proliferation	37

*Resting and regeneration refer to specimens obtained at partial hepatectomy and at the second postoperative day, respectively. Mean values of 4 protein-fed and 5 two weeks' protein-depleted rats. The 18% casein-containing and the corresponding protein-free diets were fed *ad libitum* pre- and postoperatively.

†μM thiocyanate/min./gm. dry weight at *p*H 7.3 and 20° C.

The results clearly show that rhodanese deficiency is not an invariable or necessary attribute of malignancy. Presumably, the concentration of this enzyme, as that of many other enzymes serving specialized functions, tends to decline when malignancy arises, the degree of the decrease corresponding to the degree of morphologic dedifferentation.

To substantiate this interpretation, table 2 lists the rhodanese activity of a number of spontaneous and primary induced cancers and their normal homologues. The renal carcinoma of the frog, a fairly differentiated tumor of a normal tissue as active as rat liver, was about as rich in rhodanese as the hepatomas, whereas the hypernephroid human cancer which showed little resemblance to normal kidney, gave low assays. So did the colloidal carcinoma of the stomach, presumably to a large extent because of its high content of inert matter. The fairly well differentiated intestinal adenocarcinomas displayed about the same activity as normal mucosa.

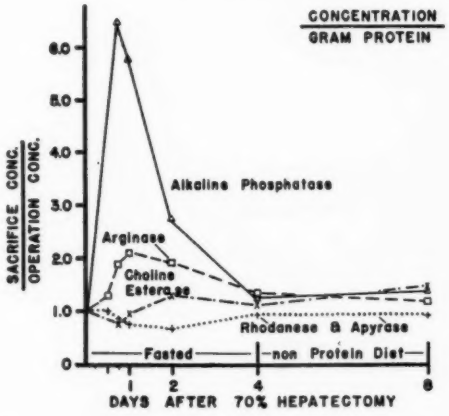
TABLE 2.—*Rhodanese activity of homologous normal and neoplastic tissues*

Species	Tissue	Normal	Neoplastic	
		Rh-units*	Type	Rh-units
Rat	Liver	534	Malignant hepatoma	142
Frog	Kidney	498	Carcinoma	191
Man	Kidney	130	Hypernephroid carcinoma.	10
Man	Rectal mucosa	68	Adenocarcinoma	54
Man	Jejunal mucosa	27	Adenocarcinoma	47
Man	Gastric mucosa	57	Colloid carcinoma	4

*μM thiocyanate/min./gm. dry wt. at *p*H 7.3; 20° C.

Gal, Fung, and Greenberg (7) found that fetal rat liver displayed only 20 percent of the rhodanese activity of normal adult liver. This discovery is in accord with the rule that the enzyme activity of cancer is usually more similar to that of embryonal tissue than to that of adult tissue. From table 1 it was evident that in adult animals rhodanese values close to the hepatoma level were brought about by the combined action of

protein depletion and regeneration. It was also observed that non-neoplastic areas from livers of hepatoma rats which received the aminoazo-dye-containing semi-synthetic diet up to the time of sacrifice, gave subnormal rhodanese assays (325 units), whereas normal values (521 units) were obtained with similar specimens from an animal with widespread malignancy following several months of realimentation with a dye-free natural diet. The enzyme depression produced by the carcinogenic diet is thus reversible as long as the cells do not become neoplastic. Text-figure 1 serves to demonstrate the reversibility of the reduction in rhodanese activity of regenerating liver from adult protein-depleted rats. It also furnishes comparative information on the regeneration patterns of other liver enzymes.



TEXT-FIGURE 1.—Regeneration pattern of hepatic enzymes. Relative specific enzyme activity per unit weight of liver protein. The specific activity of the surgically removed liver lobes has been taken as unity. Mean values of four to six experiments for each interval of time.

From the dotted curve in text-figure 1 it can be seen that rhodanese activity started to fall toward the end of the first postoperative day when protein synthesis became prominent, passed through a minimum amount during the subsequent period of most rapid protein synthesis and cell multiplication, and gradually rose when the rate of restoration slowed down. The regeneration pattern was not affected by the nutritional state of the animal (*8*). It is not a unique feature of rhodanese, for it was also followed by ATPase and tributyrinase (*9*). The regeneration of arginase, alkaline phosphatase, and nonspecific cholinesterase, on the other hand, had an entirely different time course and was greatly influenced by the metabolic state of the animal.

When liver homogenates in isotonic sucrose solution were fractionated by differential centrifugation according to the procedure of Schneider and Hogeboom (*10*), 80 to 90 percent of the rhodanese activity was recovered in the mitochondrial fraction (*11*) corroborating work of others (*12*). This held for resting and regenerating livers from both protein-fed and protein-depleted rats. Likewise there appeared to be no significant change of the specific rhodanese activity of the mitochondria. Hence the rhodanese activity level can be taken as an index of the quantity of mitochondria per unit-mass of tissue or protein. Indeed the regeneration pattern of rhodanese is in accord with that of mitochondria studied by other investigators (*13*, 14). The intracellular distribution of rhodanese in experimental hepatomas remains to be studied. It is probable that the assay of this remarkably stable mitochondrial enzyme may provide useful information on changes in the quantity and quality of the mitochondria during the neoplastic transformation of liver cells.

Summary

Data on the rhodanese activity of primary induced hepatomas of the rat are presented and compared with the rhodanese activity of homologous normal and neoplastic tissues in other species and in resting and regenerating liver tissue from protein-fed, protein-starved, and aminoazo-dye-fed rats. Activity of the hepatoma is about one fifth that of normal liver and about one half that of regenerating liver of protein-depleted rats. It is pointed out that the assay of this remarkably stable mitochondrial enzyme may provide a criterion of quantitative and qualitative changes in the mitochondria of hepatic cancer.

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Utilization of Uracil by Acetylaminofluorene-treated Rats[1,2]

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We have found that the incidence of hepatomas induced in rats by 2-acetylamino fluorene (AAF) is decreased significantly by simultaneous administration of 2-thiouraci (*1*). At the time this observation was made, the fact, demonstrated more recently (*2*), that thyroidectomy produces a similar effect, was not known; indeed, certain preliminary observations suggested that this effect of thiouracil probably could not be accounted for entirely by the associated hypothyroid state.

The experiments of Plentl and Schoenheimer (*3*) and of Bendich *et al.* (*4*) indicate that preformed pyrimidines are not precursors of nucleic acids in the normal adult rat. Uracil, however, is utilized in this way by tobacco mosaic virus, *Tetrahymena geleii*, certain bacteria, and seedlings. Thiouracil has been found to act as an antagonist to uracil and to inhibit growth of tobacco mosaic virus (*5*) and *Tetrahymena geleii* (*6*), apparently by competitive inhibition. That a similar phenomenon might be occurring in AAF-treated rats was suggested by the observation that the inhibition of hepatoma formation induced by thiouracil could be overcome by simultaneous administration of uracil (*7*). The possibility was raised, therefore, that uracil might serve as a nutritional factor for preneoplastic rat liver, in contrast to normal liver. This possibility was explored by administration of uracil-2-C^{14} to (*a*) normal rats, (*b*) rats, without hepatomas, after 90 days' administration of AAF, and (*c*) rats with hepatomas induced by AAF.

The results of these studies (*8*) indicate that preformed uracil is incorporated into the pentosenucleic acids (PNA) of AAF-induced hepatomas (as uridylic and cytidylic acids) and also, approximately to the same extent, in the liver PNA of AAF-treated rats without tumors (90 days), 80 to 90 percent of which would have developed hepatomas subsequently if they had been allowed to survive without additional AAF treatment. No significant incorporation occurred in normal rats.

The intimate relation of nucleic acids to cell growth and multiplication has led to an intensive search for evidence of qualitative differences in the nucleic acid metabolism of normal and neoplastic tissues. Our observations suggest that such a difference does exist in the case of the liver of the AAF-treated rat before the liver cells show morphologic evidence of either serious damage or neoplastic transformation. The important question as to whether this abnormal metabolic pathway operates under other conditions, *e.g.*, in regenerating or degenerating liver, is currently being investigated.

When homogenates of normal rat liver were incubated *in vitro* with uracil-2-C^{14}, there was significant incorporation into the nucleic acids (*9*), in contrast to its nonutilization for this purpose in the intact normal animal. This observation indicates that whereas normal liver cells possess enzyme mechanisms capable of utilizing uracil

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for PNA synthesis, this potentiality is not manifested *in vivo* under normal conditions. The implication is that the observed incorporation of uracil into liver PNA during AAF carcinogenesis may result from alteration of physiologic control of nucleic acid synthesis. These findings emphasize the importance of pursuing studies of metabolism in relation to carcinogenesis in the intact animal or perhaps in perfused organs, in addition to more or less isolated *in vitro* systems.

Preliminary studies with thiouracil-2-C^{14} apparently indicate that thiouracil is incorporated in the liver nucleic acids of AAF-treated rats. However, thiouracil apparently does not interfere with the incorporation of uracil into the liver nucleic acids, suggesting that the observed antagonistic influence of these compounds in relation to hepatoma formation is probably not explicable on the basis of competitive antagonism. A similar observation has been reported in the case of 8-azaguanine (*10*), which inhibits growth of Sarcoma 37, and is incorporated into the tumor nucleic acids, but does not block incorporation of guanine or of 4-amino-5-imidazolecarboxamide.

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Cirrhosis of the Liver in Rats Following Total-Body X Irradiation[1]

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We have submitted for publication some data on the production of cirrhosis of the liver following total-body X irradiation. In studying the nitrogen excretion of rats which had been exposed to 450 to 500 r total-body X irradiation, it was observed that rats ingesting a 6-percent protein (casein) diet failed to gain weight early in the post-irradiation period, whereas animals ingesting a 15- or 20-percent casein diet, and isocaloric with the 6-percent casein diet, began to gain weight rapidly 3 days following exposure to irradiation.

This failure of the rats on the 6-percent casein diet to gain weight early in the post-irradiation period led us to study the possible changes which might occur in some of the organs of these animals if allowed to continue on the diet for a prolonged period of time. Table 1 summarizes the data of our experiments.

TABLE 1.—*Cirrhosis of the liver following 450 r total-body X irradiation on various diets*

Diet*	No. of animals	No. deaths in 70 days	Number with cirrhosis of liver after 145 days
C-6	45	17	15
C-15	46	5	0
C-20	45	19	0
C-6 controls	50	0	0†

*C-6, C-15, and C-20 refer to 6-, 15- and 20-percent casein diets, respectively.
†No cirrhosis after 565 days.

Seventeen of the 45 animals on the C-6 diet died in 70 days. Fifteen of the remaining 28 rats showed marked cirrhosis of the liver. Animals ingesting the basal diet with no exposure to irradiation failed to show any cirrhosis after 565 days.

Whether the appearance of cirrhosis is a direct result of radiation exposure or the impairment of the utilization of constituents (choline, methyl groups, etc.) as a result of radiation is a subject for further investigation.

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Dr. Ralph W. Brauer, U. S. Naval Radiological Defense Laboratory, San Francisco, Calif.: In the first place I wish to congratulate Dr. White on providing for the first time convincing evidence concerning something that many of us have suspected for a number of years—namely, that far from being radiation-resistant, the liver in many ways is a target organ for ionizing radiation which fails to show evidence of histologic changes because of its extreme regenerative or recuperative powers. I should like to call attention to the fact that Dr. White's most impressive experiments were performed using Sprague-Dawley rats. In our laboratory it has been found that this strain shows a marked enlargement of the liver following exposure to ionizing radiation in doses which, on the whole, have been somewhat larger than those employed by Dr. White. Since this phenomenon has not been encountered in several other strains of rats, I wonder whether Dr. White has made observations in such other strains and whether the data indicate that there is any connection between the observed enlargement of the liver and sensitivity to the development of cirrhosis under his conditions?

In the second place, as I mentioned above, I have been impressed with the transient nature of liver injury normally seen in well-fed rats following radiation exposure. I should think it would be very interesting therefore to learn about the sequence of histopathologic events intervening between radiation exposure and the development of full-fledged cirrhosis in Dr. White's rats. I wonder if such data are available?

Effects of Cortisone on Nucleic Acid Composition of Rat Liver [1, 2]

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Cortisone, when administered intramuscularly in large doses (25 mg./day for 5 days) to rats, causes several striking changes in the composition of the liver. Most striking is the decrease in basophilia (*1–4*) when sections of such livers are stained with toluidine blue or gallocyanin chromalum. Equally striking, is a change in nucleic acid (NA) composition of the liver, and an alteration in mitochondrial numbers.

Highly polymerized ribonucleic acid (RNA) is extractable from tissue with hot salt solutions and precipitable out of such solution with ethanol (*5, 6*). As the degree of polymerization decreases, alcohol precipitation no longer occurs. Heating tissue in 5-percent trichloroacetic acid (TCA) at 80° C. also effectively extracts RNA from tissue (*7*), but this procedure depolymerizes the NA, and hence offers no distinction between highly polymerized polynucleotides and low molecular weight nucleotides.

In normal rat liver, polymerized RNA accounts for about 80 percent of the total RNA. Following cortisone administration and at a time when the total RNA per gm. of liver falls by one fourth, the polymerized fraction falls to but 32 percent of the total RNA (table 1).

TABLE 1.—*Effect of cortisone on polymerization of ribonucleic acid**

	DNA-deoxyribose (*μg./gm.*)	RNA-ribose		Polym. RNA (*percent*)
		Total RNA (*μg./gm.*)	Polym. RNA (*μg./gm.*)	
Normal	1050 ± 24. 7	4220 ± 86. 4	3320 ± 116	79
Cortisone	589 ± 13. 5	3090 ± 151	993 ± 83. 4	32
Percent change		−27	−70	

*Average and S. E. of mean of 8 experiments.

The basophilia of liver cells is normally seen in association with mitochondria, hence it seemed interesting to examine the effect of cortisone on the number of mitochondria in liver cells. Enumeration of these structures (*8*) revealed a most significant decrease in the livers of treated animals (table 2). Chemical characterization (table 3) indicated that there was an almost complete absence of polymerized RNA in mitochondria of treated livers and a significant fall in total RNA, lipid phosphorus

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and nitrogen. However, the decrease in these last three components was not so great as the decrease in mitochondrial numbers. This suggested that the remaining mitochondria were actually larger than those of normal livers, and indeed, photomicrographs (*8*) strongly suggest that this is true. It is interesting that these altered mitochondria are capable of normal oxidative phosphorylation (*9*).

TABLE 2.—*Effect of cortisone on mitochondrial numbers of rat liver cells**

	Mitochondria† $\times 10^9$	Nuclei† $\times 10^8$	Mitochondria per nucleus‡
Normal (20)	105 ± 1. 06	160 ± 22	834 ± 108
Cortisone (17)	41.8 ± 3. 9	159 ± 12	278 ± 25. 7
			$P<0.001$

*Average and S. E. of mean.
†Per gm. of wet liver.
‡Average of total number of individual determinations.

TABLE 3.—*Effect of cortisone administration upon composition of mitochondria**

	RNA†		Lipid-P†	Nitrogen†
	Total	Polym.		
Normal	908	727	271	5. 93
	± 25. 8	± 30. 5	± 24. 2	± 0. 16
No. of Expt.	(8)	(8)	(4)	(23)
Cortisone	614	34	129	4. 53
	± 53. 4	± 21. 1	± 12. 3	± 0. 53
No. of Expt.	(8)	(8)	(8)	(12)

*Mean and S. E. of mean.
†μg./gm. of wet liver.

Davidson (*10*), Ris and Mirsky (*11*) and others have suggested that the recording of measurements of intracellular constituents be related to deoxyribonucleic acid (DNA). This suggestion has been predicated upon the evidence that, except in the event of cell death, the DNA per cell remains constant. This notation is apparently not possible in the case of cortisone-treated livers, since in this case the DNA per nucleus decreases (*12*), (table 4).

TABLE 4.—*Effect of cortisone on DNA content of nuclei**

	Nuclei $\times 10^8$		DNA		
	Parench.	Total	μg./gm.	μg. $\times 10^{-8}$ per nucleus	
				Parench.	Total
Normal (8)	125	207	796	6. 36	3. 85
	± 8. 61	± 12. 4	± 12. 8		± 0. 232
Cortisone (8)	133	210	629	4. 72	2. 96
	± 1. 97	± 6. 45	± 22. 0		± 0. 10
					$P>0.003$

*Mean and S. E. of mean.

An attempt has been made to elucidate the mechanism whereby cortisone administration causes a decrease in polymerized RNA. Incubation of rat liver with cortisone

revealed no change in RNA. However, incubation of a normal liver brei with brei of a cortisone-treated liver caused a fall in total polymerized RNA as well as in the RNA of the microsome fraction (24,000 × g for 90 minutes) and the ultracentrifugable fraction (110,000 × g for 90 minutes). These data (table 5) strongly suggest the existence in the cortisone-treated rat liver of increased depolymerase or ribonuclease activity.

TABLE 5.—*Effect of incubation of liver homogenates on amount of polymerized RNA*

Material	DNA	RNA-ribose*					
		Whole Cell		Mi		U	
		T	P	T	P	T	P
Normal	797	4530	3265	1262	677	1109	831
Cortisone	514	3313	0	644	0	641	0
Mixture	650	3848	69	889	225	798	48
Percent theot. observed	99. 2	98. 1	4. 22	93. 3	66. 4	91. 2	11. 5

*μg./gm.

Summary and Conclusions

The administration of cortisone to rats in large doses causes the following changes in liver-cell composition: 1) There is a fall in total and polymerized RNA, although the fall in the latter fraction is two times as great as it is in the former. 2) There occurs a decrease in the number of mitochondria. 3) In the mitochondrial fraction there is a decrease in the nitrogen, lipid phosphorus and total RNA, and a virtual disappearance of polymerized RNA. 4) The DNA per nucleus decreases about 20 percent. 5) There is evidence to indicate the appearance in increased amounts of a depolymerase in livers of cortisone-treated animals. The appearance of this enzyme may help to explain the fall in the amount of polymerized RNA, which occurs as a consequence of cortisone administration.

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The Role of Hormones in Liver Carcinogenesis [1,2]

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Sex hormone influences in liver carcinogenesis are already well established. However, the sex pattern has not always been entirely uniform. From the results reported by Andervont and associates (*1*), female mice were more susceptible than males to the development of liver tumors following administration of *o*-aminoazotoluene. This sex difference appears in all strains studied. Intact females were more susceptible than castrate females, and castrate males were almost as susceptible as intact females. Administration of testosterone to either castrate male or female mice previously injected with *o*-aminoazotoluene resulted in fewer hepatic lesions and hepatomas (*2*). Shelton (*3*) has recently reported that strain A female mice fed diets containing *o*-aminoazotoluene developed liver tumors more readily if given stilbestrol.

In contrast to the above observations, most of the available evidence would suggest that male rats are slightly more susceptible than females to liver-tumor development from the feeding of diets containing the carcinogenic azo dyes. The most convincing data in this respect are those of Rumsfeld, Miller, and Baumann (*4*). We have tried, unsuccessfully, to establish that sex hormones or related factors would influence the hepatocarcinogenicity of the azo dyes in the rat. The following groups of albino rats were fed diets containing 0.06 percent 4-dimethylaminoazobenzene:

A—control male;
B—males given diethylstilbestrol;
C—males given testosterone;
D—control females;
E—females given diethylstilbestrol;
F—females given testosterone.

All groups were sacrificed at the same time and no significant differences were noted in any group with respect to hepatic lesions or hepatomas. In another study carried out in our laboratory, gonadectomized male and female rats were fed purified diets containing 0.06 percent 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB). Part of the gonadectomized male rats were injected with estradiol and part of the female rats were given testosterone. After 12 weeks, most of the animals were sacrificed and the liver-tumor incidence was almost identical in all groups. This study indicates that liver tumors will develop when either male or female gonadectomized rats are given the above azo compound. It was further implied that the gonads or the steroid sex hormones do not influence the carcinogenicity of 3′-MeDAB to any great extent. Any

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sex differences that do exist are inherent at a different level, perhaps at the pituitary or adrenal level.

By rather ingenious techniques, Dr. Richardson has confirmed that male rats develop liver tumors more readily than females when fed diets containing 3′-MeDAB. These experiments will be described by Dr. Richardson at the close of this presentation.

Leathem (*5*) found liver changes in 90 percent of male rats fed diets containing acetylaminofluorene (AAF); however, less than 10 percent of pair-fed female rats showed liver change. It appears to be generally accepted that male rats of at least several strains are more susceptible than females to this agent (*6*). Dr. Leathem also reported in one of the earlier sessions of this Conference that 10 of 12 male mice fed a semisynthetic diet containing AAF developed hepatomas. No tumors were found in the female mice in this study. Morris and associates (*7*), in a thorough study of the effect of both feeding and painting AAF (and several related substances) in rats, found that liver tumors developed in both sexes. It is of interest to point out that AAF and diacetylaminofluorene (dietary) produced mostly hepatomas and with approximately the same incidence in each sex. However, both cholangiomas and hepatomas resulted from the application of these substances to the skin. Only cholangiomas resulted from the feeding of diets containing 2-aminofluorene.

Allison and associates (*8*) found that hepatic neoplasms developed in 3 of 6 dogs (male) fed diets containing AAF for periods of from 6 to 9 months. Recently Morris and Eyestone (*9*) reported that 4 of 5 dogs given dietary AAF for 68 to 91 months developed liver tumors. Of the 4 dogs with hepatic tumors 2 were males and 2 were females.

There is ample evidence to indicate that hepatic tumor formation is influenced in certain species and strains by sex factors. Such influences are not consistent, however, and many of these chemically induced tumors appear equally well in the presence or absence of the gonads or steroid sex hormones. Further studies are required to establish the role of these sex factors in the mechanism of liver carcinogenesis.

Role of Thyroid in Liver Carcinogenesis

Miller and Baumann (*10*) have found that rats fed diets containing 3′-MeDAB or DAB, and given iodinated casein to increase the metabolic rate, increased the liver-tumor incidence. Administration of either thiouracil or 6-*n*-propyl-2-thiouracil, in order to produce hypothyroidism, did not alter the development of azo-dye liver tumors to any great extent. The authors were of the opinion that an increase in the BMR may augment the processes that lead to the formation of liver tumors. In our own experiments, administration of thiourea (dietary) to the rat slightly delayed the onset of liver tumors from 3′-MeDAB. This may have been more of a toxic effect, since the rats lost considerable weight and their food intake was lessened. From available data there is no really convincing evidence that thyroid function is involved in the azo-dye initiation of liver tumors. Bielschowsky and Hall (*11*) have noted that thyroidectomy inhibited liver-tumor formation in rats fed either AAF or aminofluorene.

Pancreas

Salzberg (*12*) has found that alloxan-diabetic rats did not develop liver tumors as readily as intact control animals when fed diets containing 3′-MeDAB. This is a most interesting observation since the food intakes and consequently the azo-dye intakes were very high in the diabetic rats. Carbohydrate metabolism and insulin function may thus be directly involved in azo-dye carcinogenesis.

Role of the Pituitary and Adrenals in Liver Carcinogenesis

Brief reference is made to the investigations of Moon and associates (*13–15*) wherein the prolonged administration of pituitary growth hormone (GH) to female rats resulted in the appearance of tumors in many tissues and organs. Tumors did not appear, however, in hypophysectomized, growth-hormone treated rats (*16*). Korteweg and Thomas (*17*) observed fewer tumors in hypophysectomized mice treated with 3,4-

benzpyrene than in treated controls. Implantation of methylcholanthrene in hypophysectomized rats failed to induce tumors, according to Moon and co-workers (*18*). We have obtained tumors in hypophysectomized rats injected with benzpyrene, dibenzanthracene or methylcholanthrene. The latent period for the appearance of these tumors was somewhat longer and the over-all incidence was also lower than that for intact animals.

Intact and hypophysectomized male rats of the Sprague-Dawley strain were fed purified diets containing 0.06 percent 3′-MeDAB. Animals from each group were sacrificed after 14 weeks. Severe cirrhosis and large multiple tumors were evident in the livers of the intact animals. The livers of the hypophysectomized rats were normal (*19*). After 19 weeks of dye-feeding, the livers of this latter group still remained normal. No attempt was made in this initial study to control food intakes. Since hypophysectomized animals have relatively low food intakes this could possibly have accounted for the absence of preneoplastic change in their livers. Several groups of intact rats (pair-fed) restricted to the same azo-dye diet intake as hypophysectomized rats have developed cirrhosis and liver tumors. From these and other results we feel certain that this inhibition of azo-dye liver carcinogenesis in hypophysectomized rats may be attributed to a loss of pituitary function and not to food or dye intake.

Mention should be made at this time of the special care that must be given the hypophysectomized rats maintained for long periods of time on the purified diets containing the 3′-MeDAB. The initial state of the animal is of great importance and the long time-survival appears to depend upon the technique of the removal of the pituitary. An attempt was made throughout these investigations to maintain the hypophysectomized rats as close to 27° C. as possible.

The next phase of the investigation was to determine what pituitary hormones or pituitary target glands were involved in azo-dye liver carcinogenesis. Generally, this was approached by administration of one or more hormone preparations to groups of hypophysectomized rats fed diets containing 0.06 percent 3′-MeDAB. Results obtained with a large number of hormones have already been published (*20–23*). In addition, Dr. H. L. Richardson of Sloan-Kettering Institute has carried out extensive histopathologic studies on the tissues of all these animals. (See references *20–23* and also comments pertaining to this presentation.)

A summary of the hormone studies is presented in table 1. Various ACTH preparations were found to be at least partially effective in restoring azo-dye liver carcinogenesis in the hypophysectomized rats (table 1). Complete restoration was not achieved, however, since it required approximately 21 weeks for half of the animals to develop hepatomas, while 12 weeks of dye-feeding usually results in a 100-percent incidence in intact male rats. It is of interest to note that all the liver tumors that appeared in the ACTH-treated hypophysectomized rats fed the 3′-MeDAB were diagnosed as cholangiomas. Administration of growth hormone (GH) to hypophysectomized rats also restored the azo-dye carcinogenesis to approximately the same extent as was noted with ACTH (table 1). We do not have any explanation as to why both ACTH and GH should be active in promoting azo-dye carcinogenesis in hypophysectomized rats. It is possible that only one of these hormones is active and that one preparation is contaminated with the other. However, we seriously doubt if this is the case. The possibility remains that trace amounts of an unidentified pituitary hormone or factor were responsible for the effect noted.

Other pituitary hormones (gonadotrophins, thyrotrophin) possessed only slight activity in the above respect and we must assume for the present that such activity was the result of GH, ACTH or other unidentified factors present in these preparations.

Since ACTH partially restored the azo-dye carcinogenesis in the hypophysectomized rats, adrenal function may also be implied to be involved. Administration of DOCA and corticosterone did result in the appearance of some mild precancerous changes in hypophysectomized rats, and a tumor was also identified in one of the animals treated with the latter compound. Cortisone and several other hormones had no effect in restoring the formation of hepatomas in the hypophysectomized rat (table 1). While we were unsuccessful in influencing the process with adrenal corticoids it must be

TABLE 1.—*Effect of hormones on azo-dye carcinogenesis in hypophysectomized rats.* Gross observations on livers at times of sacrifice*

Treatment†	Number of rats	Weeks of experiment	Appearance of liver
None	5	28–30	Normal
0.14 M NaCl	15	14–21	Normal
Adrenocorticotrophin (Armour ACTH-HP)	10	14–21	Cirrhosis—tumors
Adrenocorticotrophin (Equine)	13	14–21	Cirrhosis—tumors
Growth hormone (Armour)	7	14–21	Cirrhosis—tumors
Pituitary thyrotrophin	10	14–21	7 Normal, 3 cirrhosis
Synapoidin ‡	8	14–21	Normal, mild cirrhosis in some
Pranturon §	8	14–21	Normal, mild cirrhosis in some
Testosterone	9	14–21	Normal
Deoxycorticosterone acetate	11	14–21	3 Mild cirrhosis, others normal
Cortisone	6	14–21	Normal
Corticosterone	2	9	1 Normal, 1 tumor
Pituitrin	6	14–21	Normal

*Male rats of the Sprague-Dawley strain maintained on purified diets containing 0.06 percent 3′-methyl-4 dimethylaminoazobenzene.

†See references *19–23* for methods and amounts of drugs administered.

‡Synapoidin is a combination of chorionic gonadotrophin and follicle-stimulating hormone, Parke, Davis & Company.

§Pranturon is chorionic gonadotrophic hormone, Schering Corporation.

recognized that only one compound was administered to any given group of animals. Perhaps this process is dependent upon several corticoids in equilibrium. This possibility will be investigated further.

Symeonidis, Mulay, and Burgoyne (*24*) reported that adrenalectomized rats without adrenal-cortical regeneration, kept for 189 days on a synthetic diet with 4-dimethylaminoazobenzene, and with or without DOCA, did not develop liver tumors. Intact dye-fed rats given this drug also failed to develop hepatomas. The dosage of deoxycorticosterone acetate (DOCA) was 16 mg. per rat every 4 weeks as implanted pellets. Intact rats or those with regenerated adrenals developed liver tumors. In studies carried out in our laboratory, rats fed diets containing 3′-MeDAB and injected with 2 mg. aqueous suspension of DOCA or cortisone every 48 hours for 13 weeks developed cirrhosis and liver tumors. Also, adrenalectomized rats fed 3′-MeDAB developed liver tumors as readily as intact rats. We cannot account for the apparent differences; however, a more potent azo carcinogen was employed in our studies. Establishment of the role of the adrenal in liver-tumor induction is made difficult by the accessory adrenal tissues reportedly present in the rat and by incomplete adrenalectomies.

The goal of the present investigation is to restore completely azo-dye liver carcinogenesis in the hypophysectomized rat. Thus far, both ACTH and GH appear to be involved; however, the mechanisms are not apparent. GH does influence liver function and composition. Attention is called to the excellent review of Reid (*25*) on the relation of GH and ACTH to the incidence and growth of tumors.

Characteristic changes were noted in the morphology, chemical composition and metabolism of the livers of intact rats fed diets containing 3′-MeDAB. These included:

A. Decrease in the concentration of riboflavine.
B. Increase in the concentration of deoxyribonucleic acid.
C. The formation of protein-bound dye with a maximum after approximately 3 weeks and a subsequent decrease.
D. A considerable proliferation of the bile ducts after approximately 3 weeks of dye-feeding.

In the hypophysectomized dye-fed rats, the following changes were noted:

A. A slight initial decrease occurred in the concentration of liver riboflavine.
B. The concentration of deoxyribonucleic acid remained constant.

C. Protein-bound dye was formed and reached a maximum after 3 or 4 weeks. The level of protein-bound dye remained high for as long as 16 weeks of dye-feeding.
D. Bile-duct proliferation did not occur.
E. The livers of the hypophysectomized animals had approximately the same capacity to enzymatically demethylate or reduce the azo linkage as that noted for livers of intact rats. (see Dr. E. C. Miller's, earlier report presented to this Conference, p. 1571.)

From the above observations it would appear that the azo dye or its metabolites are bound in the livers of the hypophysectomized rat but that pituitary function is required in order for the process of azo-dye carcinogenesis to go to completion. It is also possible that bile-duct proliferation is under the influence of the pituitary.

The question may arise as to whether the activity of other hepatic carcinogens is lessened by removal of the pituitary. Preliminary results would suggest that diacetylaminofluorene (2-diAAF) does not induce liver or ear-duct tumors in hypophysectomized rats. Intact control animals fed diets containing this agent exhibited liver lesions and ear-duct tumors. This finding, while of interest, should not be regarded as conclusive. Several difficulties such as low food intake, 2-diAAF toxicity, etc., were encountered and further studies will be required.

Role of Hypothalamus

Slusher and Roberts (*26*) have recently isolated two materials from bovine hypothalamus capable of stimulating release of ACTH from the pituitary as judged by eosinopenia or by decline in adrenal ascorbic acid. Investigations are required in order to ascertain whether the hypothalamus is involved in the formation or growth of liver tumors.

Effect of Hormones on Established Liver Tumors

Removal of the pituitary does not have any effect on the growth of azo-dye-induced liver tumors. From the observations of others as well as those from our laboratory, hormones have little or no effect upon the growth of established liver tumors.

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Dr. H. L. Richardson, Sloan-Kettering Institute, New York, N. Y.:[1] Continuous feeding of 3′-MeDAB (0.06%) to Sprague-Dawley-strain rats from the sixth week of age produces a consistent histologic pattern of liver changes. Liver-cell change may be found as early as the fourth to the sixth week. Bile-duct proliferation and adenomas are present between the seventh and ninth week. By the twelfth week bile-duct adenocarcinomas and hepatocarcinomas are present. In a series of 54 rats, 69 percent had expired within 22 weeks and 100 percent in 29 weeks on continuous dye-feeding. In this group the malignant neoplasms of liver-cell origin were hepatocarcinomas, adenocarcinomas, and anaplastic carcinomas. The malignant bile-duct carcinomas were adenocarcinomas and papillary cyst adenocarcinomas. Malignant neoplasms of stromal origin were fibrosarcomas and angiosarcomas.

In order to study the sex differences and other factors involved in liver-tumor induction, 3′-MeDAB was administered for periods of 7, 8, and 9 weeks. This was followed by the basic diet until the animals were sacrificed at 16 weeks. Table 1 illustrates a preliminary experiment on the influence of needle trauma.

TABLE 1.—*The influence of trauma on the histologic excitation of liver cancer in male and female rats fed the azo dye 3′-methyl-4-dimethylaminoazobenzene*

Weeks on basal diet 3′-MeDAB	Weeks on basal diet	Control group* (*percent*)		Trauma administered to the liver four times following the azo dye (*percent*)	
		Male	Female	Male	Female
7	7 to 16	0	0	66	6
8	8 to 16	10	0	50	0
9	9 to 16	20	0	90	0

*Forty rats in each group—20 males and 20 females of the Sprague-Dawley strain, Charles River Farms, Mass. All animals were sacrificed at 16 weeks.

In the control group (table 1) 8 weeks of feeding 3′-MeDAB produced bile-duct carcinomas and in 9 weeks both bile-duct and hepato-carcinomas were seen. Trauma was performed by a liver-punch biopsy technique and crushed preparations showed the presence of liver cells. Histologically, the liver demonstrated an equal distribution of bile-duct and hepato-carcinoma. The cancers were adjacent to the fibrous connective tissue residual tract. Metastases, although present in the mesentery and peritoneum, were not common. In another control group the punch biopsy penetrated only the peritoneum. At 16 weeks no liver carcinoma was found in this group. In the females the livers had returned to normal. In the males minimal cirrhosis, stromal thickening, and benign bile-duct adenomas were present.

From this study the induction period in the male was postulated to be 7 to 9 weeks, and in the female 9 to 12 weeks. The sex difference is apparent.

In a previous series of experiments it was found that methylcholanthrene would prevent carcinoma of the liver if added to the diet simultaneously with 3′-MeDAR before the sixth week, and there was only partial inhibition if the methylcholanthrene was added before the tenth week. In animals which had methylcholanthrene added to their 3′-MeDAB feedings at 5, 6, and 7 weeks, trauma was performed four times during the simultaneous administration of the carcinogens, and the animals were sacrificed at 16 weeks. In this series the males had an average of 34-percent carcinoma and the females had an average of 10 percent.

In Dr. Griffin's experiments the point of induction at 7 weeks in the male fed the azo dye was further emphasized, for animals hypophysectomized before the seventh week had no liver cancer at 17 weeks. All male rats hypophysectomized after the seventh week had bile-duct carcinoma.

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A relationship was found to exist between the adrenal and the pituitary. In hypophysectomy the adrenal glands, as well as the thyroid, thymus, testes, prostate, uterus, ovaries, kidneys, spleen, and pancreas, are atrophied. A hypophysectomized rat fed 3′-MeDAB fails to develop the changes which precede liver tumor. The administration of ACTH-AR and growth hormone, separately or simultaneously, partially restores this process. This results in liver stromal changes, bile-duct proliferation, and bile-duct carcinomas in 20 to 22 weeks. Pituitary gonadotrophins and thyrotrophin were less effective in the restorative process. The impurities contained within the hormones, especially the ACTH contaminants, can be demonstrated to exist by the lipoidal restitution of the adrenal cortex. Concurrently with the lipoidal restitution, liver morphologic changes took place, resulting either in cirrhosis or bile-duct adenocarcinoma.

Cortisone, testosterone, and deoxycorticosterone acetate were ineffective (tables 2 and 3).

TABLE 2.—*Hormone preparations and dosages administered*

Group and Hormone	Dosage
Male Rats	
I ACTH-AR	2 units
II Beef growth hormone in aqueous suspension	0.5 mg.
III Thyrotrophin in aqueous suspension	0.5 mg.
IV Gonadotrophin	
Crude preparation	2.0 mg.
Synapoidin	0.5 mg.
Pranturon	0.5 mg.
V Cortisone acetate in aqueous suspension	2.0 mg.
VI Testosterone cyclopentylpropionate in sesame oil	3.0 mg.
VII Deoxycorticosterone acetate in aqueous suspension	2.0 mg.
Female Rats	
VIII ACTH-AR	2.0 units
IX Testosterone cyclopentylpropionate in sesame oil	3.0 mg.
X Deoxycorticosterone acetate in aqueous suspension	2.0 mg.
XI Pituitrin	0.05 mg.

Incidental to the study of the pituitary-adrenal relationship, it was found in the adrenal glands of intact animals receiving graduated dosages of nitrogen mustard that the outer zona glomerulosa of the adrenal cortex readily became depleted of lipoid. In contrast, it was found that a hypophysectomized rat still retained the lipoid in the zona glomerulosa regardless of nitrogen mustard administration. Likewise it was postulated that the pituitary must therefore act in the mechanism of a pituitary adrenal relationship that controls the zona glomerulosa.

This preliminary investigation requires further study into the role of the pituitary adrenal function in carcinogenesis, not only in the azo-dye study but also in the study of carcinogens that affect multiple sites in the body.

TABLE 3.*—*Effect of hormones on azo-dye carcinogenesis in hypophysectomized rats fed 3′-MeDAB. Observations on the liver and adrenal at time of sacrifice—adrenal lipoid structure graded structure according to cortical zone*

Group	8–9 Weeks										13–14 Weeks										20–22 Weeks									
	Total no. animals	Stromal change	Myeloid metaplasia	Cirrhosis	Adenomas	Bile-duct carcinoma	Adrenal-cortical lipoid				Total no. animals	Stromal change	Myeloid metaplasia	Cirrhosis	Adenomas	Bile-duct carcinoma	Adrenal-cortical lipoid				Total no. animals	Stromal change	Myeloid metaplasia	Cirrhosis	Adenomas	Bile-duct carcinoma	Adrenal-cortical lipoid			
							Glomerulosa	Transitional	Fasciculata	Reticularis							Glomerulosa	Transitional	Fasciculata	Reticularis							Glomerulosa	Transitional	Fasciculata	Reticularis
Male Rats+																														
I. ACTHAR‡	8	4	7	2	.	.	++ Restitution	+	+++	+	14	12	10	12	9	1	++ Restitution	+	+++	+	10	9	6	9	6	5	++ Restitution	+	+++	++
II. Growth hormone§	3	1	.	1	1	.	++ Part. restitution	+	+–	+	2	1	1	1	1	.	++ Atrophy	+	– – –	–+	4	4	4	4	4	3	+++ Atrophy	–	– – –	+
III. Thyrotrophin‖	2	.	.	.	.	.	++ Part. restitution	+	–+–	–+	2	.	.	.	.	.	++ Atrophy	–	– – –	–+	3	2	2	2	2	.	+++ Atrophy	–	– – –	–+
IV. Gonadotrophin																					2	1	1	1	.	.	++ Restitution	+	++	+
Crude preparation¶	7	5	5	5	1	.	++ Restitution	+	+++	+–	6	4	2	4	3	2	++ Restitution	+	+++	++										
Synapoidin**	2	1	.	1	1	.	+++ Atrophy	+–	– – –	++	3	2	1	1	1	.	++ Atrophy	+–	– – –	+–	3	2	2	2	1	1	+++ Part. restitution	+	–+–	–+
Pranturon††	2	.	.	.	.	.	++ Atrophy	+	– – –	++	2	1	1	1	.	.	+++ Atrophy	+–	– – –	+–	2	2	.	1	1	.	+++ Atrophy	–	– – –	–+
V. Cortisone‡‡	2	2	1	.	.	.	+++ Atrophy	–	– – –	+	3	.	2	.	.	.	+++ Atrophy	–	– – –	+–	.	.	.	.	.	.	.	.	.	.
VI. Testosterone	1	.	.	.	.	.	++ Atrophy	–	– – –	+	4	.	.	.	.	.	+++ Atrophy	–	– – –	+–	2	.	.	.	.	.	+++ Atrophy	–	– – –	–+
VII. DOCA	2	.	.	.	.	.	++ Atrophy	–	– – –	+	4	.	.	.	.	.	+++ Atrophy	–	– – –	+	4	3	.	3	2	.	+++ Atrophy	–	– – –	–+
Female Rats+																														
VIII. ACTHAR††	4	2	1	.	.	.	++ Restitution	+	+++	++	5	4	1	2	.	.	++ Restitution	+	+++	++	4	4	1	4	3	2	++ Restitution	+	+++	++
IX. Testosterone	1	.	.	.	.	.	+++ Atrophy	–	– – –	–	2	1	.	1	.	.	+++ Atrophy	–	– – –	–+	.	.	.	.	.	.	.	.	.	.
X. DOCA	2	1	1	.	.	.	++ Part. restitution	–	–++	++	2	2	1	.	.	.	++ Part. restitution	–	– –+	++	2	2	.	2	2	.	+++ Atrophy	–	– – –	+–
XI. Pituitrin	2	2	1	.	.	.	++ Part. restitution	–	–++	++	1	1	.	.	.	.	+++ Part. restitution	–	–++	+–	1	1	.	1	.	.	+++ Atrophy	–	– –	+–

+ Indicates lipoid was present and demonstrates its position within the zone; – indicates no lipoid in this zone of the cortex. *This table is reproduced from *Cancer* with the permission of the Editors. †Sprague-Dawley strain, Hormone Assay Laboratories, Inc., maintained on purified diets containing 0.06%, 3′-MeDAB. ‡ACTH—ACTHAR GEL, Armour & Co. §Beef growth hormone in aqueous suspension, Armour Laboratories. ‖Thyrotropin in aqueous suspension, Armour Laboratories. ¶Crude equine anterior pituitary extract containing gonadotrophin (Stanford University). **Synapoidin is a combination of chorionic gonadotrophin and follicle-stimulating hormone, Parke, Davis & Co. ††Pranturon is a chorionic gonadotrophic hormone, Schering Corp. ‡‡Cortisone acetate in aqueous suspension, Cortone Acetate, Merck & Co.

Dr. A. S. Mulay, National Cancer Institute, Bethesda, Md.: Rats of the Osborne-Mendel strain, 3 to 4 months of age, were fed diets containing 0.06 percent 4-dimethylaminoazobenzene for approximately two hundred days. At this time the liver tumor incidence was 100 percent in the males and only about 20 percent in the females. A greater sex difference is thus apparent in this strain than has been noted with other strains of rats fed this azo compound.

Dr. H. B. Andervont, National Cancer Institute, Bethesda, Md.: Dr. Griffin's excellent presentation of hormonal influences in the occurrence of hepatomas is, in fact, a continuation of previous sessions. The pronounced sex difference in susceptibility to both spontaneous and induced hepatomas should be of interest to those investigating the mechanisms involved when normal liver cells become malignant. Two inbred strains of mice (C3H and CBA) show a relatively high incidence of spontaneous hepatomas, with males more susceptible than females (*1*). There is evidence that castration or estrogenic stimulation alters the susceptibility of males (*1, 2*). When *o*-aminoazotoluene is administered to mice, especially to strain BALB/c, it is found that females are more susceptible than males to induced hepatomas (*3*). This sex response can be altered easily by castration or administration of sex hormones (*4*).

The results of transplantation presented at an earlier session were obtained during experiments designed primarily to ascertain whether strain C3H mice show any sex variation in susceptibility to hepatomas induced with *o*-aminoazotoluene or carbon tetrachloride. It was found that, as in other strains, females are more susceptible to hepatomas induced with *o*-aminoazotoluene, but males and females responded similarly to hepatomas induced with carbon tetrachloride. Thus, within one inbred strain, males are more susceptible to the occurrence of spontaneous hepatomas, and females are more susceptible to hepatomas induced with an azo dye, while the sexes are equally susceptible to hepatomas induced with carbon tetrachloride. It is hoped these variations within an inbred strain will be of assistance to those studying the mechanisms of carcinogenesis.

Speaking of carbon tetrachloride reminds one of the omission of this interesting carcinogen from the discussions of this Conference. Edwards (*5*) discovered that it induces hepatomas in mice, and he contributed much to our knowledge of its carcinogenic properties. Eschenbrenner (*6*) performed a series of excellent dose-response studies with this compound and concluded that repeated liver necrosis and regeneration may not be essential for the induction of tumors. He (*7*) also found that hepatomas can be evoked in mice with chloroform.

The ability of some compounds to elicit hepatomas following subcutaneous injection has not received much attention. A visitor who is not well acquainted with experimental hepatomas may have obtained an impression from the past few sessions that continuous oral administration is essential for the production of these tumors. This is not the case for all compounds. Early publications by Yoshida (*8*) and Kinosita (*9*) described the appearance of hepatomas in experimental animals following subcutaneous administration of carcinogens. Shear (*10*) reported similar findings. It was observed that a single subcutaneous or oral dose of 4 or 8 mg. of *o*-aminoazotoluene dissolved in 0.25 cc. of olive oil induced hepatomas in strain A female mice (*11*). This work was done to assist those who are interested in the mechanism of hepatoma formation, since large amounts of a compound may elicit tissue and metabolic reactions which are not involved in the production of tumors.

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Effect of Cortisone Administration on Induced and Transplanted Hepatomas[1,2]

CORNELIA HOCH-LIGETI, *Department of Pathology, School of Medicine, University of Virginia, Charlottesville, Va.*

The investigations presented deal with the effect of administered cortisone on the induction and transplantability of hepatomas in rats.

In the experiments with induced hepatomas, *p*-dimethylaminoazobenzene (DAB) and 2-acetylaminofluorine (AAF) were fed as carcinogens. In the first experiment with DAB 110 young Wistar rats of both sexes, weighing about 80 to 100 gm. at the beginning of the experiment, were put on a basic low protein diet (table 1). Of 90 rats fed at a level of 0.07 gm. of DAB per 100 gm. of food, 50 received 5 mg. of cortisone subcutaneously 3 times a week. Twenty rats fed the basic diet without DAB were also injected with cortisone.

TABLE 1.—*Composition of the diet*

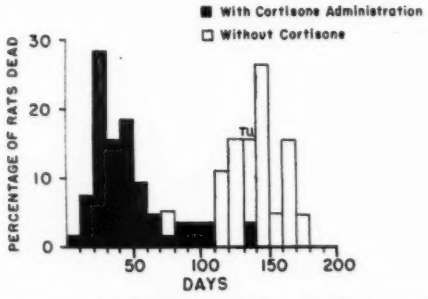
Basic diet	Percent	Vitamins for 100 gm. diet	Mg.
Vitamin-free casein	7. 0	Thiamine hydrochloride	1. 0
Sugar	6. 5	Riboflavine	. 8
Cornstarch	56. 0	Niacin	4. 0
Cellulose	12. 0	*p*-Aminobenzoic acid	10. 0
Lard	6. 5	Inositol	1. 5
Vegetable oil	8. 0	Choline citrate	10. 0
Salt mixture (U. S. P. XIV)	3. 0	Pyridoxine hydrochloride	1. 0
Cod-liver oil	1. 0	Ca-pantothenate	2. 0
		Alpha tocopherol	2. 0

Cortisone given in these doses to young rats which were fed a low protein diet proved to be highly toxic, irrespective of whether these rats received DAB or not. The combination of DAB and cortisone, however, was the most deleterious. Rats started to succumb from the 6th day on, and 80 percent of the rats receiving both cortisone and DAB died before the 60th day of the experiment. Text-figure 1 shows that the rats receiving the diet and DAB without cortisone lived considerably longer. The post-mortem examination of the rats revealed that the cause of death was either an extremely virulent pyogenic infection or hepatic necrosis. The infection, in most cases, involved the lungs, where multiple abscesses with extensive necrosis were found. Masses of bacteria were seen in these lesions. In many rats, similar though smaller abscesses were found in the myocardium, the subepicardial tissue, and the kidneys. The inflammatory areas in the kidneys were located either irregularly or in the papillae,

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 13, 1954.

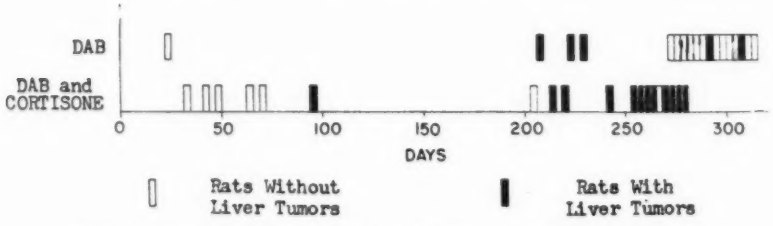
[2] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

where they were reminiscent of the necrotizing papillitis found in the human. Extensive hepatic necrosis as the cause of death was found from the 3rd week on. In the rats receiving DAB but not cortisone, many deaths occurred after the 90th day. These rats showed marked fatty changes and cirrhosis of the liver; hepatic tumors were found in two rats that died on the 135th and 137th days. Since all the rats which had received DAB and cortisone were dead at that time, the experiment was terminated.



TEXT-FIGURE 1.—Mortality rate of rats fed 0.07 percent DAB.

In a second set of rats this experiment was repeated using less DAB and a lower dosage of cortisone. Forty rats were put on the basic low protein diet containing 0.05 percent DAB. Of these rats, 20 received 5 mg. of cortisone weekly in one or in two injections; the other 20 did not get cortisone and served as controls. The mortality in these groups of rats was not unduly high. From the 8th month on rats were killed at intervals, and the experiment was terminated in the 9th month. Text-figure 2 and figure 1 show that the liver tumors in the group receiving cortisone occurred at an earlier date and were larger than the tumors in rats fed DAB without cortisone. Among the rats that survived for 90 days, hepatic tumors were found in 12 of 14 rats receiving cortisone and DAB, and in 5 of 17 rats fed DAB only.

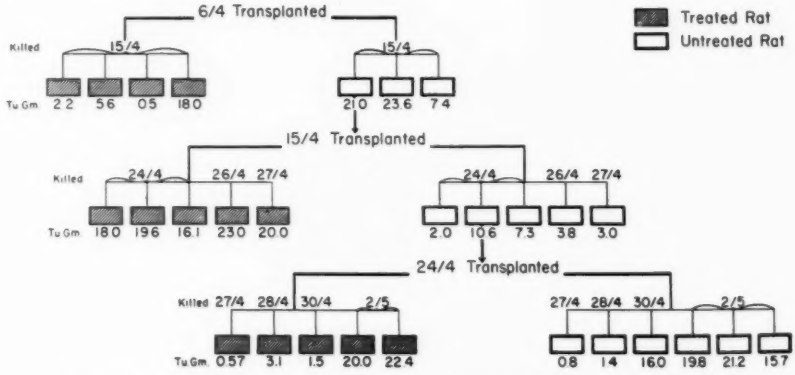


TEXT-FIGURE 2.—Occurrence of hepatic tumors in rats fed 0.05 percent *p*-dimethylaminoazobenzene.

When 2-acetylaminofluorene was used as the carcinogen, the results were similar to those found with DAB. When AAF was fed in the amounts of 0.07 percent together with a low protein diet, the mortality of the rats became extremely high on administration of cortisone. Forty rats were used in this experiment. Of the 20 rats that received cortisone and AAF, none survived 50 days. The main cause of the high mortality was found to be hepatic necrosis; in many cases abscesses of the lung were also present. An incipient hepatic tumor was found as early as the 28th day, and 5 rats that died before the 50th day had tumors in the liver. The rats which were fed AAF but not injected with cortisone showed a high mortality rate after 90 days; the experiment was terminated on the 103rd day. The first tumor in this group was found in a rat which died on the 87th day; after this time 14 of 15 rats had hepatic tumors.

It seems that cortisone accelerated the appearance of tumors induced by AAF, which was similar to the findings with the tumors induced by DAB. In order to reduce the number of tumors which develop with AAF, an experiment is in progress in which a smaller dose of only 0.03 percent AAF is being administered. In this series cortisone is given by mouth in a dosage of 0.5 mg. daily.

Experiments on the effect of cortisone on transplanted hepatomas were carried out with the transplanted hepatoma N, produced originally by Novikoff, by feeding DAB. (We are indebted to Dr. Mary E. Maver, of the National Institutes of Health, Bethesda, for the gift of this tumor.) The tumor was transplanted intraperitoneally into young Sprague-Dawley rats. This tumor grows very rapidly, and the rats usually do not survive more than 10 days after the transplantation. Five mg. of cortisone were injected subcutaneously immediately before transplantation, and then on alternate days. The arrangement of the experiment is indicated in text-figure 3, which shows that the effect of cortisone administration in 3 successive generations of transplanted tumor was not consistent. In the first transplant generation the cortisone-treated rats had smaller, and in the second generation larger, tumors than the controls. In the third set no significant effect was noted.



TEXT-FIGURE 3.—Effect of cortisone on transplanted hepatomas in rats.

Conclusions

The finding that cortisone is not well tolerated when administered to rats in which liver damage was produced emphasizes the importance of the function of the liver in the detoxification of cortisone.

The ability of cortisone to accelerate the development and increase the number of tumors induced in rats might have an important implication in human pathology. Cortisone is extensively administered to people of various ages, of whom a certain number is expected to develop tumors at some later date. Attention should be given to the possibility that cortisone might accelerate carcinogenesis.

Summary

1) The effect of cortisone on induced and transplanted hepatic tumors in rats was investigated.

2) The combination of administration of cortisone and experimental liver damage resulted in an extremely high mortality which could be attributed to massive hepatic necrosis or to fulminating infections.

3) Cortisone under certain experimental conditions seemed to accelerate the occurrence and increase the number of tumors induced by *p*-dimethylaminoazobenzene or 2-acetylaminofluorene.

4) Cortisone had no consistent effect on the transplanted hepatoma N.

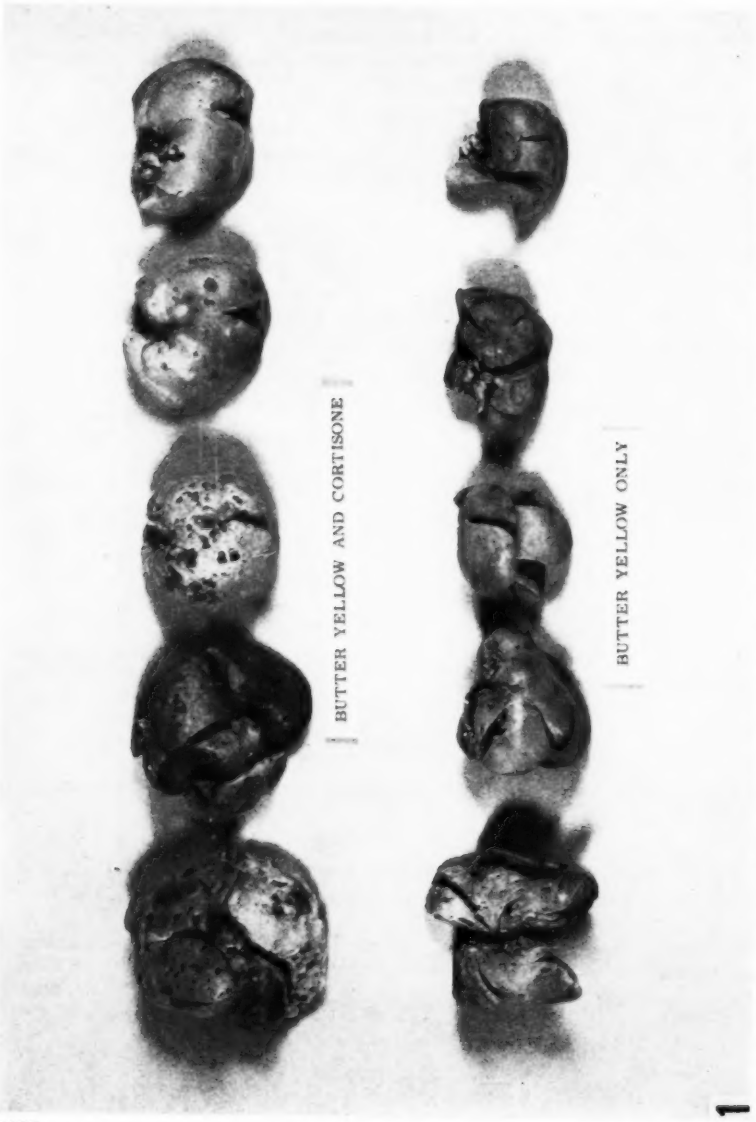


FIGURE 1.—Typical liver tumors of rats receiving butter yellow and cortisone, and butter yellow only.

Human Implications. Primary Carcinoma of the Liver in Africans[1]

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I am very grateful for the invitation to attend this Conference on Experimental Hepatomas and I am honored to appear before you in company with my friend Dr. Charles Berman, who has contributed so largely to our knowledge of liver cancer in South Africa (*1*). Like him I am chiefly interested in cancer of the liver in human beings and I have listened with interest to the many contributions to this Conference, have admired the zeal, energy and techniques which have been brought to bear on the problems of experimental hepatoma formation. But in doing so, I am only too conscious that I have been introduced to what is, to me, a new and strange world, where there live wandering tribes of pure line strains of rats, mice and other animals, migrating from laboratory to laboratory, fed on diets unknown to their ancestors, and allowed to intermarry only for special purposes under rigidly prescribed conditions. They dwell in jungles of strange synthetic chemicals. In these peculiar animals hepatomas are induced and studied, at all stages and in all varieties, with great ingenuity and by a bewildering variety of techniques. But how does all this apply to the questions with which Dr. Berman and I are concerned? Has it any relevance at all?

We have been studying hepatomatous tumors in African natives—tumors which, in their way, are as striking and as conspicuous as our African ostriches. You kindly invite me here and display a varied collection of experimentally produced hepatomas, as striking in their way as brightly colored parakeets or barnyard fowls. Birds which I admire perhaps, but surely not our familiar African ostriches. This difficulty, I am sure, arises from our different attitude and approach to the problem, and perhaps we should have here what is called, I understand in other circles, a declaration of interests. Dr. Berman and I are interested in primary carcinoma of the liver because in Africa it is a common disease, rapidly fatal, causing a great deal of human suffering and misery. We are interested in the discovery of the etiologic factors in the hope that it may be prevented. Most of you, however, are, I think, not so directly interested in primary liver cancer in humans, or in animals, except as the latter provide the means by which you can explore more deeply into the problems of the cancer cell, the essential nature of neoplasia and the mechanisms of carcinogenesis. All these are excellent aims, quite beyond criticism, and it is possible, even likely, that in your pursuit of them you may make discoveries which are directly relevant to human primary liver cancer. But in a real sense I suggest that it is irrelevant to your purposes that you are actually producing hepatomatous tumors. Your concentration upon such tumors, I suspect, arises from the variety of elegant methods with which such tumors can be produced, and the ease with which they can be studied, rather than from a direct interest in primary liver cancer in humans.

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 13, 1954.

But I am sure you are thinking that you have heard arguments like this before, probably far better expressed. Is this not a rehash of the old criticisms so often offered of experimental cancer research? This is not quite the case, however, because I think that in research into primary liver cancer, there is a danger not so apparent with researches into other forms of cancer. Most of these occur in all parts of the world; clinicians are familiar with them and can balance the observations of the experimentalists against their own clinical observations. But primary liver cancer is uncommon in the temperate regions of the world where most of the experimentalists work, whereas it is common in certain parts of the tropics where there are few experimentalists, and indeed few pathologists, interested in the problem of primary liver cancer. There are relatively few papers on liver cancer in human beings in the tropics, and much of what we know has been contributed by Dr. Berman (*1*). Under these circumstances, and because of the rapid advances in the study of experimental hepatomas, erroneous ideas get about. In pursuing your legitimate ends you have gotten, in many fields, beyond the realms of human possibility in the study of liver cancer, and the application of the knowledge you have gained to human problems, if done uncritically, may lead to confusion.

In these circumstances, I think the best contribution that I can make is to describe the problems of human primary liver cancer as I see them, with particular reference to African conditions, and perhaps to suggest to you some lines of thought which may, in the end, bring our interests closely together.

I do not think there can now be any doubt that primary liver cancer is unduly frequent in African natives, although we do not know the exact frequency. It is important, therefore, to compare the findings in Africa with those recorded elsewhere. Some excellent recent reviews by Steiner (*2*) and Edmondson and Steiner (*3*) are of great assistance in this. Now it seems to be established that the high frequency of primary liver cancer in African natives is not a racially or genetically determined phenomenon (*4*), but is probably due to environmental factors which may lead to its becoming a preventable disease (*2*). It is therefore necessary to determine as exactly as possible the circumstances in which it arises. Here we must recall that we have to consider two types of liver cancer—cancer arising from bile-duct cells, cholangiocellular carcinoma, and cancer arising from the liver cells, hepatocellular carcinoma. For brevity I shall use the terms cholangioma and hepatoma for these tumors. In passing we must surely stigmatize the iniquitous habit of some bodies who collect statistics and who insist on grouping these two types of cancer together under one heading, and strongly condemn the more iniquitous habit of grouping primary liver cancer with cancer of the gall bladder, bile ducts, or even with tumors of the peritoneum.

Before describing these tumors and their behavior in Africans we must consider the type of people in whom they occur. The native African in most areas exists throughout life on diets which are deficient in protein, especially animal protein, and in fat, but which contain much carbohydrate and fiber. The calorie intake is liable to violent seasonal fluctuations. The diets would often, by European standards, be considered deficient in minerals and vitamins, but despite this fact, it is uncommon to find clinical evidence of mineral or vitamin deficiencies. The most serious evidence of protein deficiency is seen in the weanling child. African children are usually born underweight by European standards, but for the first few months of life they thrive on the mother's breast milk, which is usually abundant. But when the mother's milk becomes insufficient for the full needs of the child and the question of supplementary feeding arises, there is no easy way in which this problem can be met. Other forms of milk are not usually available and reliance is usually placed on the adult diet suitably watered down, or sometimes pre-chewed by the mother. The child loses weight; hair and skin changes develop; it may become edematous; there is severe atrophy of the exocrine-secreting glands of the pancreas, small intestine and salivary glands, and severe fatty infiltration of the liver. This disease, kwashiorkor, is very common in many parts of Africa. Those seen in hospitals are a small proportion of the affected children; indeed in some areas few seem to escape without at least a mild

attack of the disease. Moreover, the child who survives still has to live on much the same sort of diet as that on which the disease arose (*5*, *6*).

There seem to be many serious consequences which result from this disease—in many cases permanent alterations in the enzyme-secreting glands—and there is also evidence of structural and functional damage of the liver. This takes the form of scarring of the portal triads, stellate fibrosis, with strands of fibrous tissue tending to delimit the liver lobules, causing a resemblance to the pig's liver (*7*). Almost all the African livers that I see show this change to some extent. From clinical observation and from laboratory tests there is evidence of functional liver damage in many Africans. One result of this may be the endocrine changes that are so frequently observed in male (*8*) and in female Africans (*9*). In the male there is a marked tendency to feminization, as shown by changes in many organs and systems. The causes of the changes are not entirely clear. It was suspected that they might be due to the failure of the liver to metabolize estrogens in the usual way, but recent work from Mexico (*10*) has suggested other explanations. Liver cancer in Africans arises in rural nonindustrialized communities, in people living on diets deficient in proteins and other substances, which fluctuate widely in the different seasons of the year. These people show evidence in many cases of endocrinal abnormalities. I think we must bear these points in mind in considering liver cancer in Africans.

My experience in recent years is summarized in table 1, based on autopsy studies at Mulago Hospital, Kampala. In some 3,500 autopsies we have encountered 63 primary liver cancers, of which 13 were cholangiomas.

I would draw your attention first to the age groups of the total autopsy population. The majority of autopsies are of people in the younger age groups. In fact if children under 10 years of age were excluded, the average age of the autopsied population was about 30 years. It is regrettable that the age was not recorded in so many cases but more careful investigation in recent years has not appreciably altered the average age at death of our autopsied series. The 1948 census figures showed that in East Africa only about 10 percent of the population were over the age of 45 years. There is therefore a great paucity of people in the "cancer years." Edmondson and Steiner (*3*) have pointed out that in areas where the frequency of liver cancer is high, the peak frequency occurs several decades earlier than in areas where the frequency is low. While the facts are no doubt correct, I doubt if the observation is of importance. Our studies in Uganda show that the proportion of all autopsies showing carcinoma of the liver in males does not vary significantly with age (*11*). The preponderance, therefore, of male cases in the earlier decades of life after puberty is due to the great frequency of patients in these decades and the paucity of older patients.

Edmondson and Steiner (*3*) also point out that in areas where the frequency of liver cancer is high there are few, if any, reports of primary liver cancers in infants and children, whereas in regions where the frequency is low there have been many reports of primary liver cancers in infants or children (*12*), including some where the cancer seems to have been of congenital origin (*13*). The absence of reports of this tumor in African children may be due to lack of observation or recording, but I have not seen a case myself, none is recorded in our autopsy series, and I know of no case in any part of Africa I have visited. There is here perhaps a field in which further observation is necessary and where the available evidence may give us some etiologic clues.

The sex ratio of carcinoma of the liver is variously reported in the literature. Edmondson and Steiner (*3*) give a male to female ratio of 1:0.6. Our experience in Kampala would suggest that the male to female ratio may be as high as 6:1, but this is perhaps misleading, as African women come to the hospital in far lesser numbers than do men. You will notice the disparity in the total autopsy figures between the two sexes. The fact that many reports of the disease in Africa have been based on such groups as soldiers (*14*), mineworkers (*1*) or other preponderantly male groups has perhaps given rise to erroneous conclusions and has exaggerated greatly the male preponderance. A recent Kampala survey revealed 43 male cases and 13 female cases, forming 9.34 percent of all male cancers and 3.89 percent of all female cancers,

TABLE 1.—*Autopsy findings in Kampala*

Age	11–20	21–30	31–40	41–50	51–60	61–70	70+	Adult	Old	Total	Grand total
No. of males	248	844	670	355	164	57	9	615	5	2, 974	
No. of females	84	188	113	48	11	5	3	18	1	599	3, 573
Postnecrotic scarring											
Males	5	23	15	12	4	1	0	15	0	75	
Females	1	4	1	0	1	0	0	0	0	7	82
Laennec cirrhosis											
Males	12	35	36	16	5	4	2	12	2	124	
Females	1	8	1	1	0	0	0	5	1	17	141
Cholangiocellular carcinoma											
Males	0	4	0	3	0	0	0	1	1	9	
Females	0	1	2	1	0	0	0	0	0	4	13
Hepatocellular carcinoma											
Males	5	16	11	6	4	0	0	4	0	46	
Females	0	0	3	1	0	0	0	0	0	4	50

respectively (*15*). When we come to consider cholangiomas and hepatomas separately, however, there will appear some differences in the two sexes.

Before doing so, however, there is the question of cirrhosis to be considered. Table 1 shows that in the 3,573 post mortems there were 223 cases of frank cirrhosis without cancer. Of the 63 cases of primary liver cancer, no cirrhosis was found or recorded in 15 cases. There were thus 271 cases of cirrhosis (7.5% of all autopsies), of which 48 were associated with carcinoma. Carcinoma thus arose in 17 percent of the cases of frank cirrhosis. The term "frank cirrhosis" is used to differentiate from the other cases in which some degree of excess fibrous tissue is almost always seen. The close association of carcinoma with cirrhosis is such that the probability of this being a chance association is infinitesimal. This association also suggests that powerful carcinogenic agents are operating in African livers with cirrhosis, as compared with experiences elsewhere, as in other countries where between 3.4 percent and 10 percent of patients with cirrhosis develop primary liver carcinoma (*1*). Edmondson and Steiner (*3*) give a figure of 5 percent. But before concluding that African cirrhotic livers are particularly and peculiarly liable to develop liver cancer, the question of hemochromatosis and liver cancer must be explored. Incidentally I have not yet encountered hemochromatosis in an African, nor in Uganda do we see the cases of pigmentary cirrhosis so frequently encountered in South Africa. The relationship between hemochromatosis and carcinoma has been discussed recently by Warren and Drake (*16*) and by Edmondson and Steiner (*3*). The former workers, using only reports where at least five autopsies had been performed on patients with hemochromatosis concluded that carcinoma supervened in 18.9 percent of the cases of hemochromatosis. The latter, using the Los Angeles records gave a figure of 13 percent, but combining their figures with those of Warren and Drake, reached a final figure of 18 percent. This is not so very different from our experience in Kampala, or of that of Kouwenaar (*17*) in the East Indies. Thus both in the cirrhosis of Africans and the cirrhosis of hemochromatosis there is much the same liability to develop a primary liver carcinoma, but this liability is distinctly higher than in the non-hemochromatosis cirrhoses of Europeans.

When we come to examine the type of cirrhosis in Africans which is associated with carcinoma, some interesting features emerge. The commonest type of cirrhosis seen in Africans is the Laennec type, which conforms quite well to that seen in other parts of the world. The other major type of cirrhosis is that which appears to be the result of a postnecrotic scarring. In my experience this occurs with a high frequency in Africans. The distinctions between these two types are not always clear but a full consideration of many sections will often enable a distinction to be made (*18*). While a good deal of discussion could be given to this matter, all I can say is that the use of the usual criteria produces the table shown. In their age distribution the two types of cirrhosis are similar. When we relate the cirrhosis to the carcinoma, however, we have the following table:

Table 2.—Relation of cirrhosis to liver carcinoma in Africans

	Cholangioma		Hepatoma	
	Male	Female	Male	Female
With no cirrhosis recorded	0	0	2	3
" " " histologically	6	3	0	1
" Laennec cirrhosis	3	0	36	0
" Postnecrotic scarring	1	0	7	0
" Schistosomal cirrhosis	0	1	1	0
Total	10	4	46	4

There is, I think, much of interest in this table. First, there seems to be a relatively low association of cholangioma with cirrhosis. For instance in 10 of 13 cases cirrhosis was excluded by microscopic examination. On the other hand, the association of

hepatoma with cirrhosis in males was almost invariable, at least cirrhosis was present in 44 of 46 cases, whereas in females the relationship was inverse. Probably none of the 4 females with hepatoma had any cirrhosis. Recent clinical experience tends to support this autopsy experience.

This being the case, I think we must examine the status of cholangiomas more closely. In Africans these tumors, in my experience, run very true to type. They are quite distinctive tumors, pale pink-white in color, firm, not bile-stained except where there is the jaundice of obstruction, rather slow growing, slow to metastasize, not subject to necrosis. In all these ways they contrast sharply with hepatomatous tumors, which are yellow-red in color, soft, always show necrotic areas, are invariably bile-stained, are rapidly growing and metastasize readily, and even the metastases are slightly bile-stained. In my experience liver cancer in Africans falls clearly and distinctly into these two types, readily distinguishable by the naked eye (*11*). Mixed tumors are quite uncommon, and in contrast to the findings of the experimentalists, bile-duct cystadenomas and hepatic cell adenomas are very rare. In fact, I have not seen a single case, although rarely small areas of cholangiofibrosis have been found. I am by no means sure that there is any greater frequency of cholangiomas in Africans than in other peoples, and I understand that Berman shares these doubts (*1*). We certainly need more information on this point, and I think we need much more information about cholangioma as a tumor. In the literature, the association of cholangioma with cirrhosis is variously quoted as from 0 percent to 100 percent. I would suggest that more reports are needed on cholangiomas in human beings, with particular reference to the association with cirrhosis and with proliferative lesions of the biliary system.

Thus the problem of primary liver cancer in Africans would appear to be the problem of the frequency of hepatomas in Africans, and experimental methods which lead to the frequent production of mixed tumors and cholangiomas seem perhaps to have little relevance.

Now in Uganda the relationship of cirrhosis to hepatoma formation in males is extremely close. In African females an opposite relationship seems to obtain, although our evidence on this point is not yet adequate. It will be interesting to see if this apparent lack of relationship of cirrhosis to carcinoma is supported by further studies, and if any differences are found before and after the menopause. Meanwhile there appears to be a striking disparity which requires explanation, and an explanation if found might take us a long way in our search for etiologic factors. In the male the type of cirrhosis encountered is usually a Laennec type of cirrhosis, chronic in nature. This is of course an old observation (*19*). This observation, I suggest, throws a shadow of doubt on the long-held belief that the hepatomatous growth is the result of excessive hyperplasia. If this were the case, would we not expect to see hepatomas arising frequently in association with the great regeneration often seen in postnecrotic scarring and perhaps in the earlier stages of a Laennec cirrhosis? Is there, in fact, solid evidence for the sequence: necrosis—regeneration—hyperplasia—neoplasia? If this were in fact the sequence of events in humans, would we not frequently encounter the mixed tumors, the adenomas, cystadenomas and cholangiomatous tumors which are seen in experimental animals, and which in fact we do not see in human cancers in Africans?

The decisive nature of the cirrhosis in human liver cancer of males seems to be of cardinal importance. What does this cirrhosis do to the liver which encourages hepatomas to develop? I do not think it is of direct importance in causing functional liver damage. We have ample evidence that severe degrees of cirrhosis can exist without evidence of functional liver damage, and we have ample evidence that severe functional liver damage can occur in Africans over long periods without cirrhosis. So to me the close association of cirrhosis and hepatomas in males seems to indicate some factor other than liver damage *per se*. Could it be that cirrhosis is primarily an expression of a time factor; that in African patients and in cases of hemochromatosis, fibrosis of the liver occurs from an early age and in a severe form, providing a background against which various carcinogenetic factors may operate?

These are in brief the circumstances in which carcinoma of the liver occurs in Africans, and in a full understanding of the etiology and pathogenesis of carcinoma of the liver all of them must be accounted for. The cardinal problem in Africans is the etiology of purely hepatocellular carcinoma. What light have your experimental findings thrown upon this problem? This is too hard a question for me to answer here. Some general observations may, however, be pertinent. We have not found in Africans any great frequency of tumors of the alimentary tract or of the urinary tract (*15*). If some specific carcinogen is involved in Africans, it appears to be one that is fairly specific to the liver cells. Such a postulated carcinogen must be one that arises or can be encountered in African life and which can explain the curious geographic distribution of carcinoma of the liver. Those suggested so far seem to be too ubiquitous or not ubiquitous enough. It has to be explained how this postulated carcinogen could operate differently in the two sexes, and why cirrhosis is so important in males. Such liver carcinogens as you are using experimentally may one day be found, singly or collectively, to fit the bill. But I am sure that this will be achieved only if we have close co-operation between the experimental cancer researchers and those of us who have the opportunity of seeing many cases of this truly dread disease in humans. In the absence of such co-operation erroneous ideas may be promulgated and accepted because the experimentalists are so far in advance of clinical pathologists, and so prolific of ideas, as compared with the relatively few pathologists who are in a position to see many cases of human liver cancer. I am, therefore, most grateful for the invitation to be here and to join in your discussions and wish to thank those institutions and individuals who have made my visit possible.

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Human Implications of the Work on Experimental Hepatomas[1]

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As a clinician interested in pathology, it is my belief that the main purpose of experimental cancer research is the better understanding of the cancer problem in man.

I have assumed, therefore, that the first objective of a research worker who elects to study cancer of a specific organ in experimental animals (*e.g.*, primary liver cancer) is the production of malignant tumors, which, by their clinical manifestations and pathologic characteristics, are identical with those originating in similar organs in man.

At this Conference, however, no attempt was made by any of the speakers to describe the clinical course of their hepatoma-bearing animals or birds. This omission is to be regretted, for, in order to assess the human implications of these experimental hepatomas, it is desirable to know whether the induced liver tumors also produced those local and general signs and symptoms of malignancy which in man are known to result from cancerous changes within the liver, from their metastases to remote organs, or from complications (*1*).

Information is necessary as to whether (and to what extent) loss of weight, emaciation, anemia, progressive liver enlargement, jaundice, dilatation of superficial abdominal veins, ascites, edema, or hematemesis (the common symptoms of human primary liver cancer) occur also in the experimental animals. What do the liver-function tests reveal? Are there instances of acute abdominal hemorrhage from the sudden rupture of a tumor nodule or erosion of a blood vessel? Do the animals develop obvious metastatic lesions in situations remote from the primary growth in the liver, as so frequently happens in human cases? It is required to know also whether liver cancers can be readily induced in very young animals, for in the Bantu races of Africa and in Indonesians, primary liver cancer often occurs at an unusually early stage in life—sometimes before the age of 20 or 30 years.

The histologic features of the hepatomas induced with 2-acetylaminofluorine or with choline-deficient diets most closely resembled those of human primary liver cancers. On the other hand, the pathology of many of the other induced hepatomas (as shown at the Conference) was unfamiliar to me: the histologic appearances seemed to differ radically from human liver cancers not only in the cytologic structures, but also in respect of their architectural patterns, which in man are characteristic for this form of malignancy.

[In order to clarify these differences, the salient macroscopic and microscopic features of human primary liver cancer were then demonstrated with the aid of a series of colored lantern slides, which had been prepared from South African Bantu material. These included the cellular structure of the tumor cells (fig. 1); the characteristic architectural patterns of "hepatocellular" and "cholangiocellular" cancer; the mode of intrahepatic spread by proliferating tumor emboli (fig. 2); the regression of tumor

[1] Presented at the Conference on Experimental Hepatomas, Arden House, Harriman, N. Y., October 13, 1954.

columns by central necrosis, fatty or scirrhous changes; the relatively frequent incidence of extrahepatic metastases in distant organs or situations, where the characteristic cellular and architectural patterns of the primary liver tumor are so faithfully reproduced and where even the capacity to produce bile is often retained.]

Since so many of the experimentally induced hepatomas failed to show the pathologic features resembling (in my opinion) human liver cancer, and since there was a lack of information on the clinical manifestations of the hepatoma-bearing animals or birds, it was impossible adequately to assess the human implications of the tumors presented at this Conference.

Let me hasten to add, however, that this does not minimize the importance of the Conference. Some of the experimental studies presented have yielded valuable information on etiology. The fact that it took Dr. Morris 6 years to produce the liver tumor in a dog by means of 2-acetylaminofluorene, is not without significance insofar as human liver cancer is concerned, if one takes into account the lifespan of his experimental animal.

Again, the liver tumors induced by Drs. Salmon, Copeland, and Burns in rats and chickens fed on choline-deficient diets, and the accompanying hemosiderosis in the livers and other organs, may have a counterpart in man. Recent evidence is mounting that faulty diets, especially diets deficient in first-class proteins and in vitamins, are perhaps among the most important factors in the production of cirrhosis and primary liver cancer in the impoverished people of Africa and certain parts of Asia, among whom the incidence of those diseases is relatively high (*2*). Moreover, large amounts of iron pigment (hemosiderosis) are likewise frequently found in the livers and other tissues of Bantu cases of cirrhosis and primary liver cancer (*1*, *3*).

Further discussions on lines similar to those conducted at this Conference are obviously required. However, detailed clinical and pathologic studies, conducted by the experimental pathologist throughout the whole induction period of the hepatomas, are also urgently necessary so as to ensure that the evoked liver tumors are unequivocally identical with those in man both in regard to the local malignant process and to the general systemic effects on the host.

Such studies will necessarily require the close co-operation and integration of work with clinicians and pathologists who are experienced with the human form of primary liver cancer. Then only will it be possible to form a correct perspective on the human implications of the many outstanding contributions which experimental pathology has already made to this very important field of cancer research.

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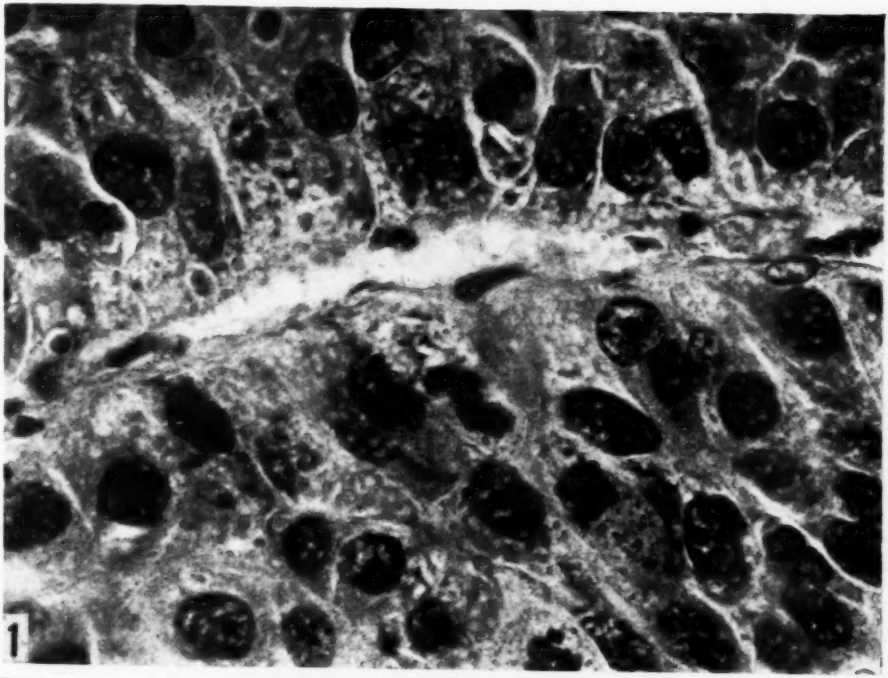
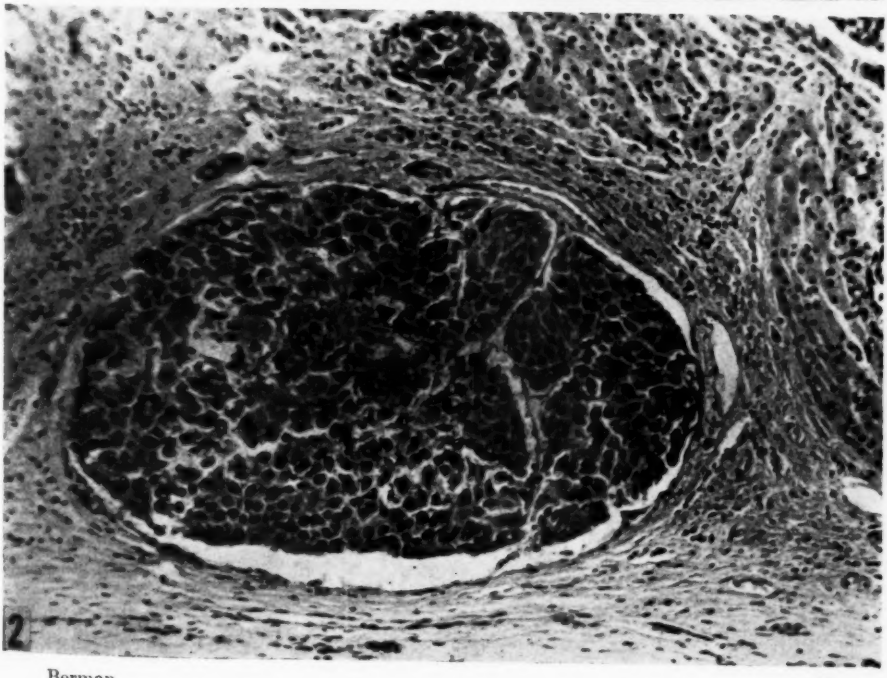
Plate 138

Figure 1.—Hepatocellular carcinoma, showing polygonal tumor cells, prominent hyperchromatic nuclei, multiplicity of nucleoli, and mitotic figures in adjacent cell columns bordering a vascular space. Note Kupffer cells. × 700

Figure 2.—Section of hepatocellular carcinoma through a cirrhotic tract in the liver, showing two organizing tumor emboli within portal vessels. × 100





Berman

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Dr. Alex B. Novikoff, Waldemar Medical Research Foundation, Port Washington, N. Y.: Most of us would agree that a cleavage does exist between animal experimentalists and clinicians. But, like cleavage of the egg which leads to an integrated organism, we hope this cleavage, too, will yield a harmonious entity. Whether experimentalist or clinician, the same goal motivates us—the speedy and effective attack upon the disease.

Dr. Shelton is quite correct in pointing to the decisive importance of instituting proper diet for prevention of liver cancer among the African peoples of whom Dr. Davies and Dr. Berman have spoken so eloquently. It might be indicated that our conviction based on comparisons of the frequencies of liver cancer encountered in different populations by clinicians is very much strengthened by the results of experimentalists with dietary effects on liver carcinogenesis in rats. Furthermore, there will remain instances of human cancer in which fuller understanding of the disease process is required. For this, animal work will be essential.

Much has been made, in the discussion here, of apparent differences between the disease in man and animals. If they exist, the study of such differences may well reveal much of importance for the understanding of cancer in man.

Finally, may I suggest that the integrated attack upon the disease will be helped if experimentalist and clinician developed closer relationships—in their institutions, scientific societies, and journals. Joint discussions through these media will more readily clarify the meaning of the experimental data for the treatment of the cancer patient, and will more effectively direct the experimentalist toward problems urgent to the clinician.

Dr. Hans Popper, Cook County Hospital, Chicago, Ill.: As a pathologist who devotes the major part of his time to the study of human diseases and only his spare time to animal experimental studies, I find myself between the viewpoints so well expressed by Drs. Davies and Berman and those exhibited otherwise in this most interesting Conference on Experimental Hepatomas. Let me first stress that the lesions observed in Africa, and so clearly and beautifully presented by Dr. Berman, are more or less identical with those which we see in this country. On the basis of a survey of almost 100 cases of primary carcinoma of the liver autopsied at Cook County Hospital in Chicago and a large number of concurring studies from various American institutions, everything which has just been presented by Dr. Berman can be confirmed.

I just want to stress two points of classification. One deals with the borderline between hepatocellular carcinoma and bile-duct carcinoma. As repeatedly stressed here, the smallest bile ducts (the ductules or cholangioles) develop embryologically from liver cells and not *vice versa*, and the same may take place in postnatal life—in both instances possibly due to stimulation by adjacent connective tissue. It is therefore not surprising that almost every (human or animal) hepatocellular carcinoma reveals cholangiomatous features. The presence of such features, therefore, should not deter us from designating the lesion as a hepatocellular carcinoma. As a matter of fact, it appears that liver cells with tendency to cholangiomatous transformation are characteristic of hepatocellular carcinoma and we have relied, for the diagnosis of hepatocellular carcinoma in liver biopsy specimen, upon the presence of structures that are on the borderline between hepatocellular plates and cholangiolar ducts. The whole group of hepatocellular carcinomas with or without more or less extensive cholangiomatous elements has biological characteristics in common; namely, tendency for invasion of the portal vein branches, marked male predominance, and frequent coincidence with cirrhosis. In contrast, the very rare bile-duct carcinoma without any hepatocellular elements does not invade the portal vein and has no sex predominance or relation to cirrhosis. To draw the line between hepatocellular carcinoma and bile-duct carcinoma, independent of the presence of cholangiomatous features, may appear, therefore, more in keeping with the biologic behavior.

The second point concerns the term *hepatoma*, which previously was often applied

to benign hepatic neoplasms. If hyperplastic or regenerative nodules, for instance in cirrhosis, are excluded (as they should be), benign hepatocellular tumors are extremely rare in human beings. It may therefore appear appropriate to reserve the term *hepatoma* to the unquestionable hepatocellular carcinoma, without including, at least in human beings, its benign counterpart which can seldom be clearly identified; then it can be designated, to avoid confusion, as hepatocellular adenoma. In closing, I would like to stress that I have received much stimulation as a pathologist dealing with human disease during the proceedings of this Conference from the attempt to go beyond mere description of the human lesion to an understanding of pathogenesis and correlation of function and structure.

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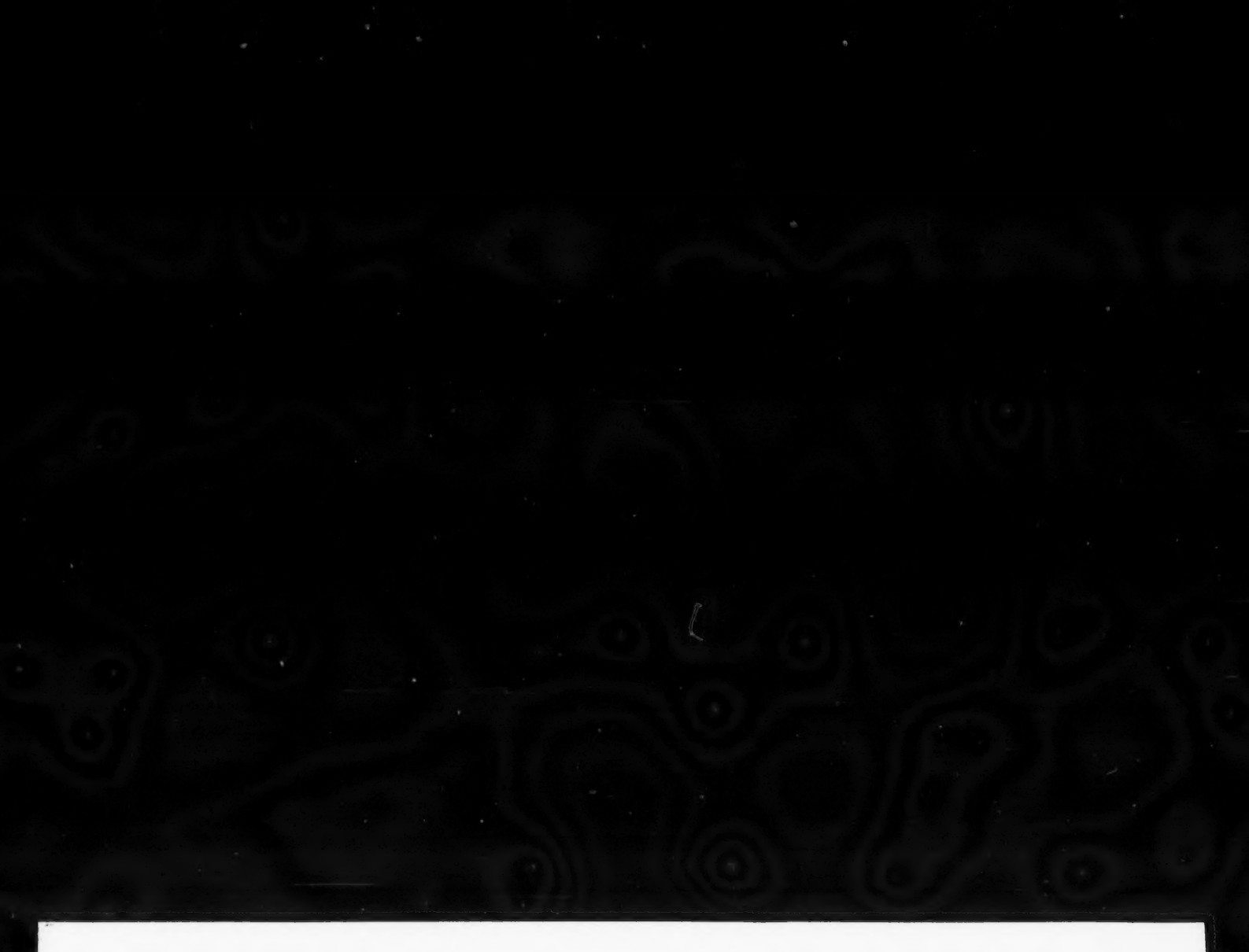
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